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Award Number: DAMD17-99-1-9298

TITLE: A Novel Approach to Increase Breast Cancer Chemosensitivity: Disruption of the Anti-Apoptotic Function of Translation Factor eIF4E

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REPORT DATE: October 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2003	Final (1 Oct 1999 - 30 Sep 2003)

4. TITLE AND SUBTITLE
A Novel Approach to Increase Breast Cancer Chemosensitivity: Disruption of the Anti-Apoptotic Function of Translation Factor eIF4E

5. FUNDING NUMBERS
DAMD17-99-1-9298

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The objective of the project was to experimentally test the idea that targeted disruption of the anti-apoptotic function of the translational complex eIF4F can sensitize breast carcinoma cells to therapeutic doses of a non-genotoxic cytostatic agents and/or to low concentrations of genotoxic agents. We demonstrated that enhanced integrity of the cap-dependent translational complex eIF4F cells breast carcinoma lines harboring diverse oncogenic alteration is associated with increased resistance of cancer cells to drug-induced apoptosis. Enforced activation of eIF4F in mammary epithelial cells increases their resistance to apoptosis and results in cells that are able to form transformed foci *in vitro*. Accordingly, targeted disruption of the cap-dependent translational complex by ectopic overexpression of the translational repressor 4E-BP1 stimulates apoptosis, abrogates chemoresistance and *in vivo* tumorigenicity in breast carcinoma cells in a manner dependent on a phosphorylation status of 4E-BP1. These data show that both genesis and maintenance of a drug resistant neoplastic phenotype in mammary epithelial cells are strictly dependent upon the activation status of the eIF4F-mediated translational apparatus. They provide the conceptual basis for a new approach to anticancer therapy: correcting aberrant translation as a complement to strategies that target malignant cells by activating apoptosis.

14. SUBJECT TERMS
Breast cancer, translational control, apoptosis, chemotherapy

15. NUMBER OF PAGES
39

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The functional activity of the translational complex eIF4F is invariably elevated in a broad spectrum of naturally occurring tumors, including breast carcinoma (Raught et al, 2000). Culture- and tumor xenograft-based experiments demonstrate that aberrant activation of eIF4F stimulates cell cycle transit (Raught et al 1999b), rescues cells from apoptosis (Polunovsky et al., 1996, 2000, Tan et al., 2000) and synergizes with pre-neoplastic alterations, such as deregulated c-Myc, in promoting malignant transformation (Raught et al, 2000). Consistent with this, the eIF4F antagonist, translational repressor 4E-BP1, displays attributes of a tumor suppressor. It is functionally inactivated in aggressive breast carcinomas by hyperphosphorylation. These findings support the idea that an aberrantly activated cap-dependent translation apparatus may be a critical determinant of breast carcinogenesis, and that the 4E-BP family of translational repressors may serve as tumor suppressors that are required for breast cancer surveillance. The objective of this awarded project is to experimentally test the idea that targeted disruption of the anti-apoptotic function of eIF4E can sensitize breast carcinoma cells to therapeutic doses of non-genotoxic cytostatic drugs and/or to low concentrations of genotoxic agents. We also propose to develop treatment strategies, which will include disruption of the anti-apoptotic function of eIF4E in a combination with treatments with non-toxic doses of conventional anti-neoplastic agents.

BODY

Experimental Data in Support of Aim 1

Aim I: *In vitro*, examine whether levels of eIF4E expression determine chemoresistance in human breast cancer cell lines.

A. Determine whether susceptibility to drug-induced apoptosis in different human breast cancer cell lines correlates with cellular levels of eIF4E protein.

B. Develop breast cancer cell lines with increased or decreased function of eIF4E and determine: 1.Whether constitutively or inducibly overexpressed eIF4E will suppress apoptosis in drug-susceptible breast carcinoma cell lines. 2. Whether breast cancer cell chemoresistance can be abrogated by a reduction of eIF4E function following ectopic expression of: (a) eIF4E antisense RNA; (b) eIF4E repressor protein 4E-BP1.

UNPUBLISHED DATA

Aim 1A. Determine whether susceptibility to drug-induced apoptosis in different human breast cancer cell lines correlates with cellular levels of eIF4E protein.

Although gain and loss of translation initiation activity potently modulates viability in Myc- and Ras-transformed rodent fibroblasts in our published studies, it remains an open question whether translational control is relevant to regulation of apoptosis in naturally occurring breast cancer cells which are of epithelial origin and posses diverse tumor-related gene alterations. To detect whether susceptibility to spontaneous and drug-induced apoptosis in different human breast cancer cell lines correlates with activity of the cap-dependent translational machinery, protein drug-induced apoptosis assays have been performed in a set of human breast carcinoma cell lines (Table 1), the genetic profile of which have been documented previously (Sepp-Lorensino et al., 1995).

Table 1. Cell lines in use in our studies

Cell line	Ras status	Other characteristics	Cell line source
HMEC 184 A1	wt	Immortalized breast epithelial cells	Berkeley National Laboratory
MDA-MB-231	Ki-V12	ER-	ATCC
MDA-MB-453	wt	ER-/erbB2+++/MAPK+++	ATCC
MCF-7	wt	ER+/IGF-IR +++	Dr. Yee, UM Cancer Center
MDA-MB-468	wt	ER-/EGFR+++	Dr. Yee, UM Cancer Center
SkBr-3	wt	ER-/HER2+++	Dr. Yee, UM Cancer Center

The Ras status, estrogen (ER) dependence, and other characteristics of non-transformed breast epithelial cells and breast carcinoma lines are shown. One cancer cell line (MDA-MD-231) harbors mutated Ki-Ras, while others express activated upstream effectors of eIF4E signaling pathways.

Extracts from non-transformed breast epithelial cells and breast cancer cells lines were tested to detect cellular levels of eIF4E and 4E-BP1, and to evaluate the association of eIF4GI with eIF4E to form an intact translation initiation complex (Figure 1). Steady state levels of eIF4E were similar among the breast cancer cell lines and only modestly increased compared to the non-transformed 184 A1 breast epithelial cells (Figure 1a). In contrast, steady state levels of eIF4G1 were significantly increased in all breast cancer cell lines tested. While 4E-BP1 is predominantly represented in non-transformed cells by hypophosphorylated isoform a which actively represses translation, breast cancer cell extracts are enriched with slow migrating hyperphosphorylated 4E-BP1 (isoforms β and γ) which is much less active in repressing assembly of eIF4F. Consistent with this, breast cancer cell extracts manifested increased amounts of eIF4GI associated with eIF4E in the cap bound fraction (Figure 1b). This indicates increased amounts of intact eIF4F complex in all breast cancer cell lines tested, suggesting these cells function in a translationally activated state.

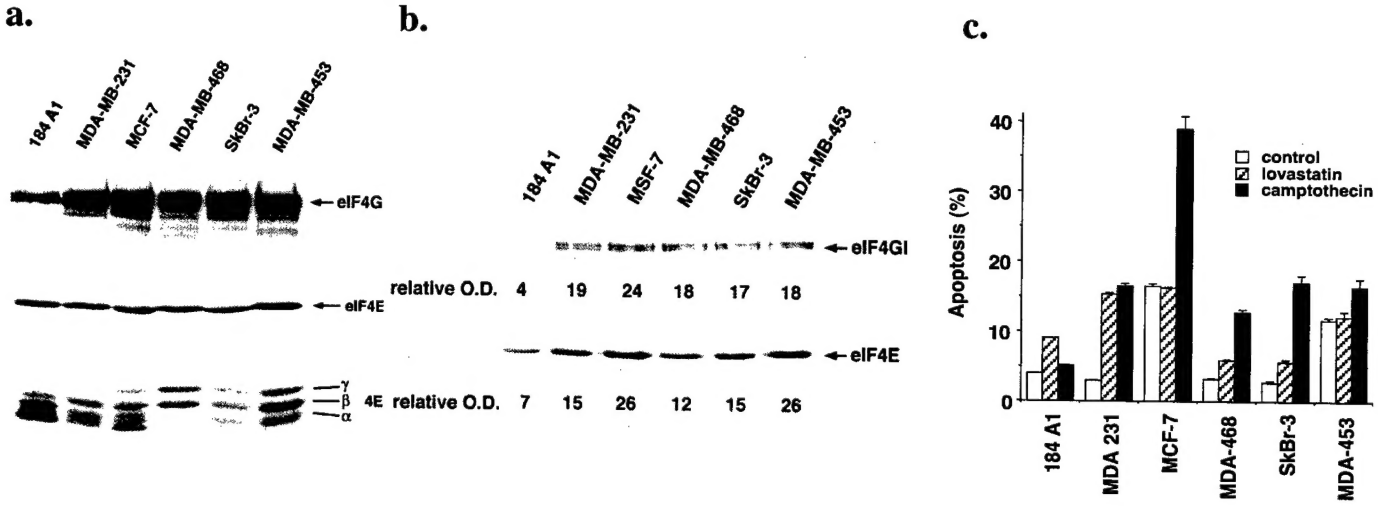


Figure 1. Selective activation of the eIF4F translation complex in breast cancer cells. **(a)** Western blot of cellular eIF4E, eIF4G1, and 4E-BP1. **(b)** Immunoblot analysis of eIF4GI associated with cap-bound eIF4E. For the cap-affinity assay, cell lysates (250 ug) were incubated with 7-methyl-GTP Sepharose resin (Amersham Pharmacia Biotech) to capture eIF4E and its binding partners. Samples were eluted with buffer containing 70 μM 7-methyl-GTP. Cap bound material was subjected to SDS PAGE and transferred to nitrocellulose. Blots were probed for eIF4E (mouse monoclonal antibody, 1:500, Transduction Laboratories), and for eIF4GI (rabbit polyclonal antibody, 1:4000). **(c)** Cells were incubated in the presence of 7.5 μM lovastatin or 500 nM camptothecin for 24 h, and percentages of cell with hypodiploid DNA contents (% of apoptosis) were quantified by flow cytometry Each bar represents the mean ± SD (4 independent replications)..

Apoptosis assays revealed elevated spontaneous apoptosis in MCF-7 and MDA-MB-453 cells as well as increased susceptibility to drug-induced apoptosis in all tested breast carcinomas (Figure 1c). Since upregulated cap-dependent translation antagonizes apoptotic death (Polunovsky et al, 1996; Tan et al., 2000), we hypothesized that breast cancer cells require a high level of cap-dependent translation to suppress the apoptotic apparatus that is activated in the course of cell malignant transformation.

Aim 1B. Develop breast cancer cell lines with increased or decreased function of eIF4E ...
Overexpressed eIF4E in immortalized HMECs confers a pre-neoplastic phenotype through activation of eIF4F

We performed a series of in vitro and in vivo experiments to explore the consequences of sustained eIF4F activation, examining its impact on malignant conversion and on maintenance of the transformed phenotype. We tested whether overexpressed eIF4E, the rate-limiting component of eIF4F, is able to transform immortalized HMECs. Early passage immortalized 184-A1 HMECs do not exhibit the hallmarks of in vitro transformation. They lack the ability to form colonies when sparsely plated in growth factor-restricted (deprived from isoproterenol and transferrin) medium on a solid substratum, or to grow in an anchorage-independent fashion in soft agar. They are also unable to induce xenograft tumor growth in vivo. 184-A1 cells (passage 48) were infected with replication-defective retrovirus contained a green fluorescent protein (GFP) gene linked to a sequence encoding hemagglutinin (HA) epitope-tagged human eIF4E (HA-eIF4E). Cells expressing the highest levels of GFP (the brightest 10% of the GFP positive cell population) were sorted using a FACSscan and propagated for further investigation Introduction of HA-eIF4E enabled HMECs to form anchorage-dependent transformed foci in medium lacking isoproterenol and transferrin, with a clonogenic potency comparable to that of MDA-MB-231 breast carcinoma cells (Figure 2a). Moreover, 184-A1/HA-eIF4E cells formed colonies in soft agar, but in contrast to breast carcinoma cells, theywere unable to form tumors in nude mice (not shown).

To detect whether ectopic eIF4E confers transformed phenotype through activation of eIF4F, 184-A1/HA-eIF4E cells were transfected with a vector bearing a neomycin resistance cassette and sequences

encoding HA-tagged wild type 4E-BP1 or its 54-63 deletion mutant (4E-BP1Δ) – which lacks the eIF4E binding site and is therefore unable to sequester eIF4E. After 48-h incubation in complete medium, transfected cells were shifted into medium containing G418 and neomycin-resistant colonies were scored. Both mock-transfected 184-A1/eIF4E cells and cells bearing 4E-BP1-Δ yielded full size colonies after 15 days. (Figure 2b). In contrast, overexpression of wild type 4E-BP1 nearly eliminated the ability of 184-A1/eIF4E cells to form colonies in selective medium. Thus, 4E-BP1 reversed the ability of eIF4E to enable HMECs to form transformed foci - in a manner dependent upon the ability of 4E-BP1 to bind and sequester eIF4E.

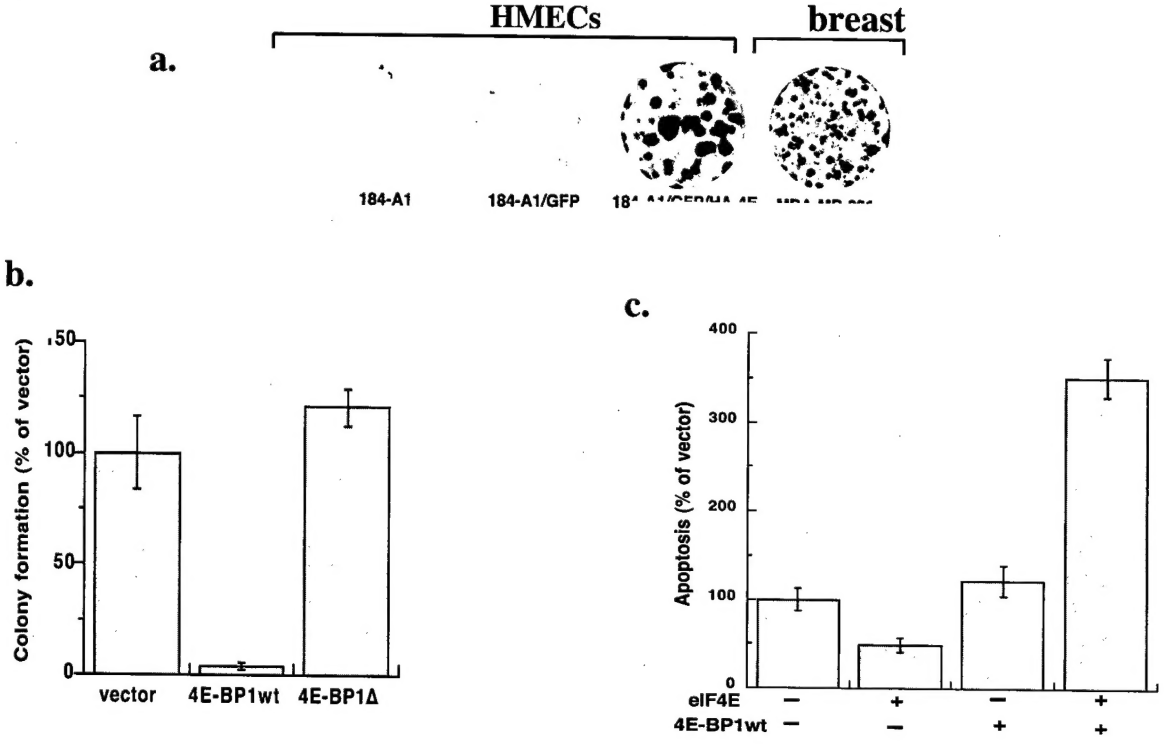


Figure 2. Overexpressed eIF4E increases survival of non-transformed HMECs and confers the ability to form transformed foci in a 4E-BP1 inhibitable manner.

Loss of colony forming ability can result from blockade of cell cycle transit and/or activation of cell death. When 184-A1 cells expressing physiological or enhanced levels of eIF4E were transiently transfected with wild type 4E-BP1 and cultivated at low density in complete growth medium, there was no effect on cell cycle distribution (data not shown), but there was a marked changed in cell viability (Figure 2c). Under these conditions, about 25% of non-transduced 184-A1 cells were apoptotic. Introduction of HA-eIF4E rescued about half of the cells from apoptosis (12.8 % ± 1.7 compared with 25.8% ± 3.2). Transfection with wild type HA-4E-BP1 (35-40% transfection efficiency as determined by GFP vector fluorescence) did not change the frequency of apoptosis in 184-A1 cells expressing eIF4E under physiological control. However, it did result in a more than 3-fold increase in the apoptotic frequency of 184-A1/eIF4E cells. **These results show that over expressed eIF4E conferred human epithelial cells with some aspects of malignant conversion, including resistance to intrinsic apoptosis and the ability to form anchorage-dependent transformed foci – properties that could be neutralized by 4E-BP1. Moreover, these results show that over expressed 4E-BP1 shifted ectopic eIF4E signaling from inhibition to activation of apoptosis.**

PUBLISHED DATA

Li S, Takasu T, Perlman D, Peterson M, Burrichter D, Avdulov S, Bitterman P, Polunovsky V. **Translation factor eIF4E rescues cells from Myc-dependent apoptosis by inhibiting cytochrome c release.** *J.Biol.Chem.* 2003, 278: 3015-3022.

Results of this study provide evidence that eIF4E-mediated rescue from oncogene-dependent apoptosis is accompanied by inhibition of mitochondrial cytochrome c release. Experiments achieving gain and loss of function demonstrated that eIF4E-mediated rescue is governed by pretranslational and translational activation of *bcl*-x, as well as by additional intermediates acting directly on - or upstream of - the mitochondria. Thus, these data traced a regulatory pathway controlling cell apoptotic susceptibility that begins with the activity state of the protein synthesis machinery, and leads to interdiction of the apoptotic program at the mitochondrial checkpoint.

Experimental Data in Support of Aim 2

Aim 2. *In vitro*, develop a novel protocol(s) for breast carcinoma treatment that will include pharmacological inhibitors of eIF4E activity and non-toxic doses of anti-cancer drugs.

UNPUBLISHED DATA

Translational repressor 4E-BP1 suppresses colony formation in MDA-MB-231 breast carcinoma cells and cooperates with camptothecin in inducing apoptotic death.

The function of eIF4E is inhibited by members of the family of translational repressors, the eIF4E binding proteins (4E-BPs, also known as PHAS) (Gingras ei al., 2001). When hypophosphorylated, 4E-BPs compete with eIF4G for binding to eIF4E and sequester eIF4E in a non-functional complex. Upon hyperphosphorylation, 4E-BPs dissociate from the complex with eIF4E allowing it to form an active translation initiation complex. To detect whether 4E-BP1 decreases viability and chemoresistance of breast cancer cells, MDA-MB-231 breast carcinoma cells were transfected with a vector pACTAG-2 engineered to encode a *neo* resistance gene cassette and haemagglutinin (HA) tagged human wt 4E-BP1(Gingras et al., 1999b). Transfected cells were incubated in the presence of G418 and numbers of neomycin resistant colonies were scored after three weeks.

Figure 2.

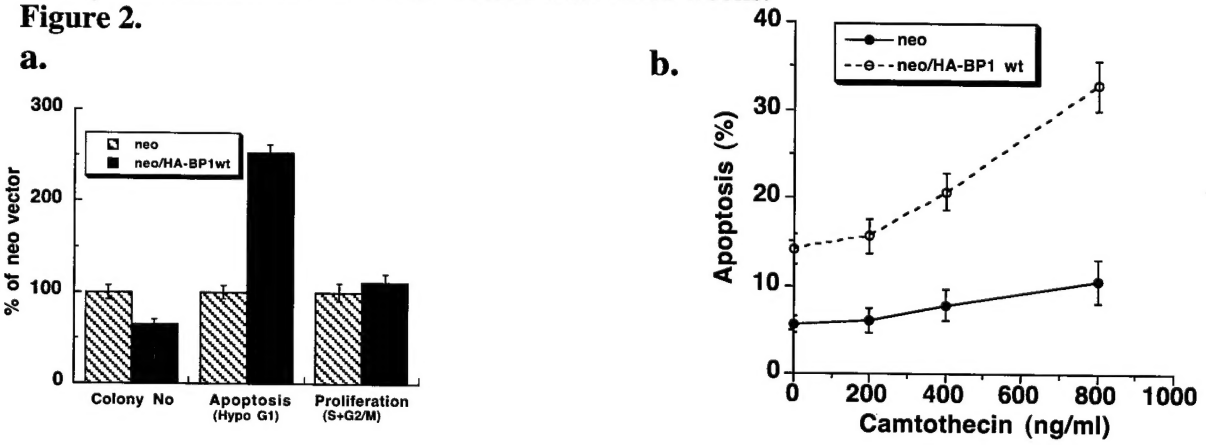


Figure 2. Ectopic 4E-BP1 decreases viability and chemoresistance in MDA-MB-231 breast carcinoma cells. (**a**) Quantative analysis of colony forming ability, apoptosis, and cell proliferation in transfected cells. (**b**) Dose-dependent effect of camptothecin on apoptosis.

The results revealed decreased capacity to form colonies in cells transfected with 4E-BP1 as compared to the cells transfected with the empty neo vector (Fig. 2a). The reduction of colony formation observed could have resulted from inhibition of cell cycle transit, activation of cell death, or both. Flow cytometric analysis of HA-4E-BP1 transfected cells revealed no changes in the S + G2/M fraction of cycling cells, whereas the fraction of hypodiploid apoptotic cells was significantly increased (2b). 4E-BP1 cooperated with anti-tumor agents camtothecin (Fig 2c) and doxorubicin (data not shown) in

induction of apoptosis. However, it was only marginally effective in promoting the proapoptitic function of lovastatin or paclitaxel. **Thus, these data show that enforced overexpression of wild type 4E-BP1 in breast cancer cells is associated with increased cell susceptibility to apoptosis. They also suggest that 4E-BP1-induced repression of cap-dependent translation selectively sensitizes breast cancer cells to anti-cancer drugs and that camptothecin and doxorubicin are most promising partners of anti-eIF4E interventions in anti-tumor therapy.**

Mutating phosphorylation sites increases the pro-apoptotic potency of exogenous 4E-BP1. To comprehensively examine the impact of 4E-BP1 phosphorylation state on growth of malignant cells, we quantified the colony form efficiency of MDA-MB-231 breast carcinoma cells transfected with a neomycin resistance cassette bearing sequences for wild type 4E-BP1 or Ser/Thr ((S/T) to Ala (A) mutants at the phosphorylation sites indicated (Fig. We employed three mutant forms of 4E-BP1: one double mutant (A37/A46) to eliminate phosphorylation N-term to the eIF4E binding site, and two single mutants A65 and A70 at phosphorylation sites C-terminal to the eIF4E binding site. To detect any potential experimental bias resulting from systematic differences in ectopic wild type mutant 4E-BP1 expression due to self repression (i.e. negative feedback from the ectopic protein itself leading to decreased translation of ectopic and/or endogenous 4E-BP1 despite identical gene transfer efficiency), cells were transiently transfected in parallel with a pACTAneo vector encoding wild type or mutant HA-tagged 4E-BP1 usi identical procedures in four independent experiments. Immunoblot analysis of 4E-BP1 expression showed simila for endogenous 4E-BP1 and all forms of HA-tagged 4E-BP1 except cells transfected with A70. For this mutant, endogenous and exogenous 4E-BP1 were reproducibly expressed at a lower level (Fig. 3b). Replication of this experiment employing a retroviral gene transfer procedure led to similar results (not shown), confirming the repressive potency of the A70 mutant for MDA-MB-231 cells.

Figure 3.

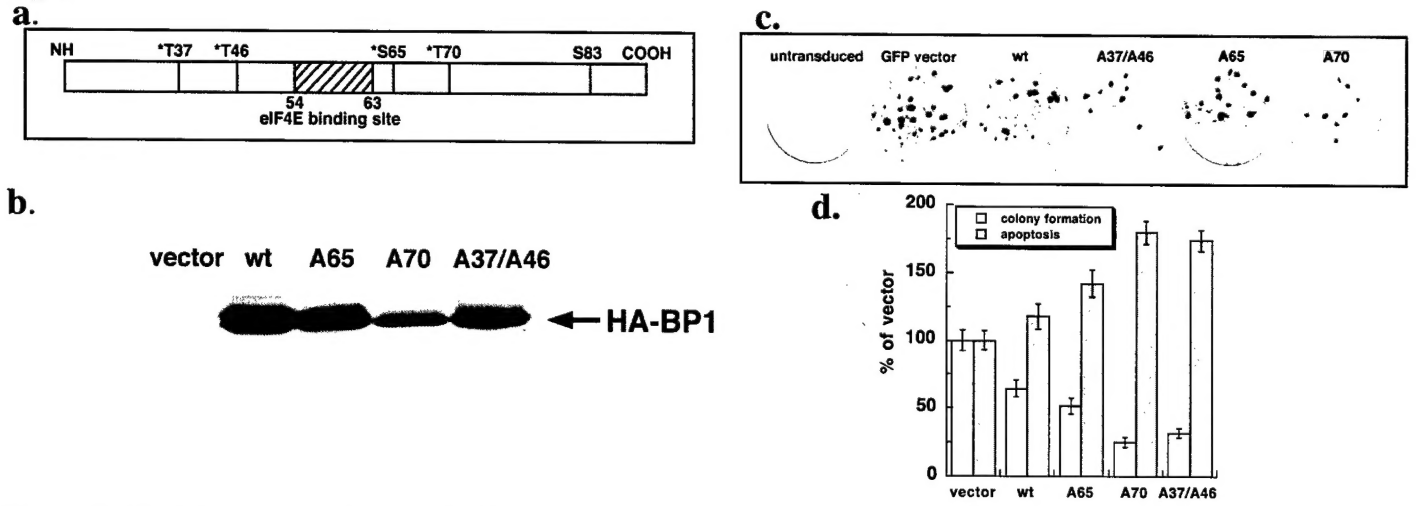


Figure 3. 4E-BP1 suppresses MDA-MB-231 breast cancer cell colony formation capacity in a manner dependent on its phosphorylation status. (a) Shown are positions of the four serine/threonine sites of 4E-BP1 mutated to alanine (designated by asterisks) relative to the eIF4E-binding region. (b) Expression of exogenous 4E-BP1 in MDA-MB-231 cells transduced with wild type or mutant 4E-BP1. (c) Photo of breast cell colony formation. (d) Colony formation abilities and frequency of apoptosis in transduced cells.

Measurements of cap-dependent and IRES-driven translational activities (performed by using a bicistronic luciferase reporter construct, demonstrated reduction of cap-dependent translation rates in cells ectopically overexpressing HA-4E-BP1 (data not shown). Mutating phosphorylated sites increased an ability of 4E-BP1 to repress translation with 75-85% inhibition of cap-dependent translation by the A70 and double A37/A46 mutants. As shown at the Fig. 3, expression of both wild type and mutant 4E-BP1 reduced MDA-MB-231 colony formation efficiency, ranging from 20% fewer colonies in response to introduction of wild type 4E-BP1, to 75-80 % reduction of colony number after transfer of A70 or

A37/A46 mutants. **Together, these results indicate that mutating phosphorylation sites increases the pro-apoptotic function of 4E-BP1 and that the rank-order of proapoptotic potency of each 4E-BP1 phosphorylation site mutant matches its potency in repressing cap-dependent protein synthesis.**

PUBLISHED DATA

Li S, Sonenberg N, Gingras AC, Peterson M, Avdulov S, Polunovsky V, Bitterman P. Translational control of cell fate: Availability of phosphorylation sites on the translational repressor 4E-BP1 governs its pro-apoptotic potency. *Mol Cell Biol.,* 2002, 22: 2853-2861.

We employed two complimentary approaches to determine whether intensifying repression of cap-dependent initiation by dephosphorylation of 4E-BP1 could trigger apoptosis in normal and oncogene-transformed fibroblasts: (1) pharmacological blockade of ectopic and endogenous 4E-BP1 phosphorylation with rapamycin; and (2) ectopic expression of 4E-BP1 mutants lacking specific phosphorylation sites. We found that inhibition of 4E-BP1 phosphorylation by rapamycin triggers apoptosis in fibroblasts ectopically expressing wild type 4E-BP1. We also showed that transient or stable expression of phosphorylation site mutants in non-transformed fibroblasts potently activates apoptosis in a phosphorylation-site specific manner, but not in strict accord with their ability to repress cap-dependent translation.

Summary of Data in Support of Aims 1 and 2

1. Our data suggest that both the apoptotic and translational machinery are activated in all tested breast carcinomas when compared to non-transformed breast epithelial cells.
2. Targeted disruption of the cap-dependent translational complex by ectopic overexpression of the translational repressor 4E-BP1 stimulates apoptosis and abrogates chemoresistance in breast carcinoma cells harboring diverse oncogenic alteration. In addition, expression of 4E-BP1 phosphorylation site mutants potently activates apoptosis in a phosphorylation-site specific manner, which parallels repression of cap-dependent translation. These results demonstrate a close connection between cellular functions controlled by 4E-BP1 and the regulation of apoptosis.
3. Contrary to our expectations, rapamycin neither stimulate apoptosis nor potentiate 4E-BP1- or drug-induced cell death. Molecular bases of the differential effect of rapamycin of transformed fibroblasts and breast cancer cells remain to be clarified. Most importantly from a therapeutic point of view, these data suggest that, at least in breast carcinomas, pharmacological normalization of the upregulated apoptotic machinery can be achieved more effectively by disrupting association of eIF4E with capped mRNAs than by interfering with the eIF4E-to-eIF4G binding. Based on these data, we plan to employ novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).
4. To develop optimal regimens for treatment of pre-sensitized breast cancer cells, we explored the role of overexpressed 4E-BP1 in apoptosis induced by a variety of cytostatic agents. We found that both wild type and hypophosphorylated 4E-BP1 sensitize breast carcinoma cells to the topoisomerase I inhibitor camptothecin and conventional anti-tumor antibiotic doxorubicin. Conversely, only marginal effects were observed when lovastatin and paclitaxel were applied. These data show that targeted disruption of cap-dependent translational machinery selectively sensitize breast carcinoma cells to a subset of anti-cancer drugs, and that doxorubicin and the topoisomerase I inhibitors are promising candidates for collaboration with anti-eIF4E pre-treatments in suppression of breast tumor growth.

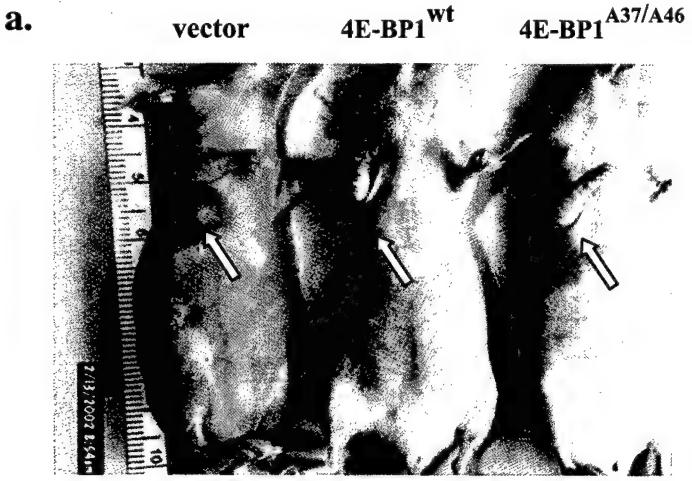
Experimental Data in Support of Aim 1

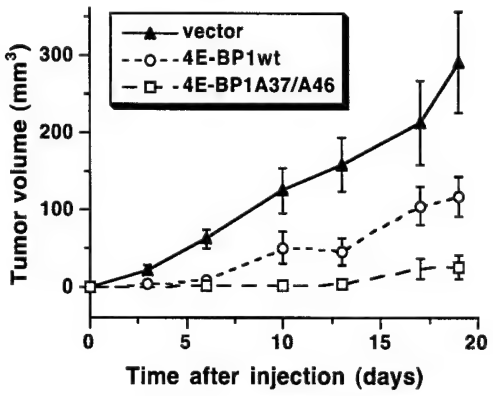
Aim 3. Utilize a nude mouse xenotransplantation preclinical model to examine proof of principle: reduction in eIF4E activity chemosensitizes human breast cancer cells.

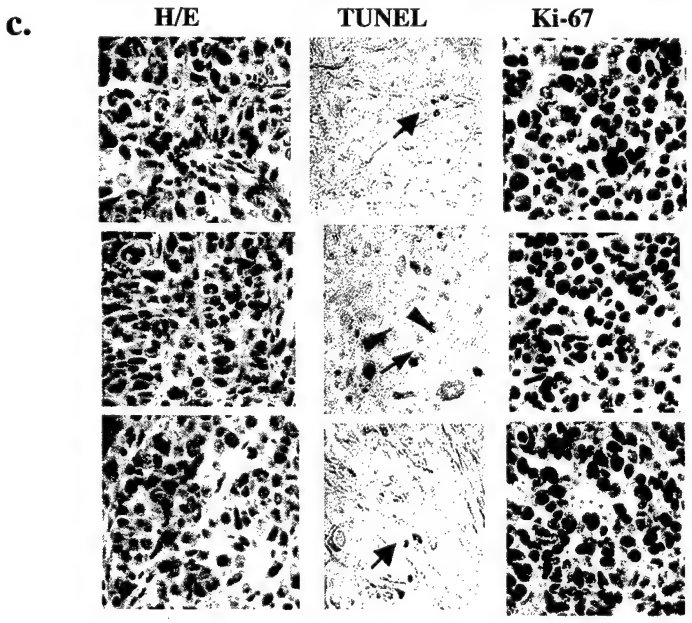
UNPUBLISHED DATA

Tumorigenicity of breast carcinoma cells is inhibited by over expressed 4E-BP1

When MDA-MB-468 cells were implanted into mammary fat pads of immunodeficient mice, they formed tumors with a latency period of 3 days, and achieved a diameter of 9.7 ± 0.8mm (mean ± SEM) within 3 weeks (Figure 4a and 4b). In marked contrast, MDA-MB-468 cells expressing high-levels of either 4E-BP1^{wt} or 4E-BP1$^{A37/A46}$ formed tumors with a much longer latency. Tumors formed by 4E-BP1^{wt} cells were first apparent at day 5, whereas 4E-BP1$^{A37/A46}$ expressing cells formed visible tumors only after twelve days. Control tumors grew at a rate 22.2 ± 1.5 mm^3/day from day 13 to day 21 after implantation; whereas during the same time period, tumors formed by 4E-BP1^{wt} cells grew 11.8 ± 1.2 mm^3/day, and tumors arising from 4E-BP1$^{A37/A46}$ cells grew very slowly - only 3.5 ± 0.7 mm^3/day.







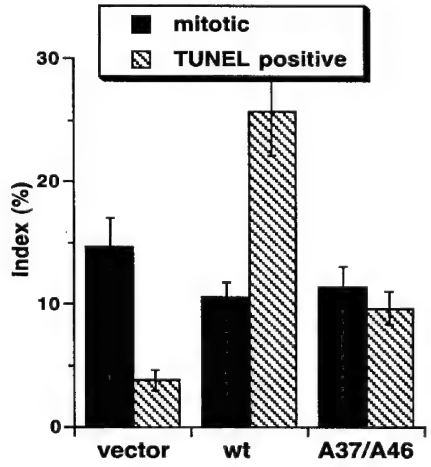


Figure 4. 4E-BP1 inhibits xenograft tumor growth in a manner dependent on its phosphorylation status.

5 x 10^6 cells from 4E-BP1 wild type (4E-BP1^{wt}) and phosphorylation site double mutant (4E-BP1$^{A37/A46}$) transduced MDA-MB-468 cell lines were injected into mammary fat pads of nude mice and evaluated for tumor growth at the indicated intervals. **a:** Representative animals photographed at 21 days postinjection. Arrows indicate loci where cells were injected. **b:** Quantification of tumor volume with time. The average tumor volume for six animals of each tested group was determined (mean ± SE). **c:** Histopathology of fad pads in mice injected with HA-4E-BP1 expressing cells. Fad pad samples were excised at time of euthanasia. Sections were stained with hematoxilin-eosin (H/E, left), or underwent TUNEL staining to assess apoptotic cells (middle) and Ki-67 immunostaining to reveal proliferating cells (right). Representative fields are shown. Arrows point to apoptotic cells and arrowheads indicate apoptotic bodies.
d: Mitotic and apoptotic frequencies in tumors formed by 4E-BP1 and vector transduced MDA-MB-468 cell lines. the results represent mean values ± SE from 500 microscopic fields of 3 tumors formed by cell expressing empty vector or HA-4E-BP1.

All tumors were excised after euthanasia at day 21. Histopathological analysis revealed that tumors formed by both 4E-BP1^{wt} and 4E-BP1$^{A37/A46}$ cells displayed increased cellular and nuclear pleomorphism and had fewer blood vessels than control tumors (Figure 4c). To evaluate these differences in tumor growth rates, tumor cell proliferatve and apoptotic indices were quantified. Proliferative activity was assessed by staining with the proliferation marker Ki-67 or quantified by counting mitotic figures on hematoxylin-eosin stained sections. Although mitotic indices in tumors that arose from 4E-BP1 overexpressing cell were slightly decreased, they exhibited a pattern of Ki-67 staining similar to those in control tumors (Figures 4c and 4d). To assess the frequency of apoptosis, tissue was analyzed using a TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. The apoptotic frequency was increased almost 7-fold in tumors arising from 4E-BP1^{wt} cells, and about 2-fold in tumors formed by 4E-BP1$^{A37/A46}$ cells (Figure 4c and 4d). **These data suggest that 4E-BP1 suppresses tumor growth primarily by promoting tumor cell apoptosis rather than by inhibiting cell proliferation.**

These data also uncovered a striking contradiction between the proapoptotic potency of 4E-BP1 forms in vitro and in vivo. Specifically, wild type 4E-BP1 displayed low level proapoptotic activity in vitro, but was highly potent in vivo. In contrast, 4E-BP1$^{A37/A46}$ manifested high apoptotic potency in vitro, but only modest activity in tumors. A clue to the significance of these findings came from an examination of tumor cell morphology. Cells ectopically expressing 4E-BP1$^{A37/A46}$ manifested a pattern of cell death that clearly differed from those in 4E-BP1^{wt}tumors. Most apoptotic cells in tumors formed by 4E-BP1^{wt} cells exhibited morphology typical for early stages of apoptosis. In contrast, the TUNEL-positive cells in tumors arising from 4E-BP1$^{A37/A46}$ cells contained predominantly compact apoptotic bodies – a morphology typical of late stage apoptotic degradation (Figure 4c). **Based on this, we considered the possibility that a cohort of HA-4E-BP1$^{A37/A46}$ cells had already undergone apoptosis during the latent period and that the relapsed tumors arose from those cells that escaped apoptosis - presumably through the loss or inactivation of 4E-BP1$^{A37/A46}$, due to strong selective pressure against this proapoptotic protein.**

Propagation of breast carcinoma cells selects for gain of apoptosis resistance and loss of hypophosphorylated 4E-BP1 expression

To assess the association between ectopic expression of 4E-BP1 and tumor cell death, tumors formed by 4E-BP1^{wt} and 4E-BP1$^{A37/A46}$ cells were evaluated for HA-4E-BP1 expression by HA immunostaining. Both 4E-BP1^{wt} and 4E-BP1$^{A37/A46}$ tumors revealed high levels of ectopic 4E-BP1, predominantly in the regions where intensive cell death was observed (Figure 5a). Of note, the expression of HA-4E-BP1 in viable cells of 4E- BP1$^{A37/A46}$ tumors was markedly reduced compared with that found in tumors formed by 4E-BP1^{wt} cells. This suggested the possibility that the process of tumor formation indeed selected for cells that evade apoptosis by loss of hypophosphorylated forms of 4E-BP1, and that the re-growth of 4E-BP1$^{A37/A46}$ tumors after a long latency resulted from loss of ectopic 4E-BP1 expression.

To approach this possibility experimentally, we traced expression of exogenous 4E-BP1^{wt} and the phosphorylation site mutants in long-term cultures of non-transformed HMECs, and breast carcinoma cells. In HMECs, which resist the pro-apoptotic effects of all forms of 4E-BP1 tested, expression levels

of ectopic 4E-BP1 were unchanged during the entire period of observation (Figure 5b). In striking contrast, MDA-MB-468 cells revealed a dramatic reduction in steady state levels of the mutant forms of

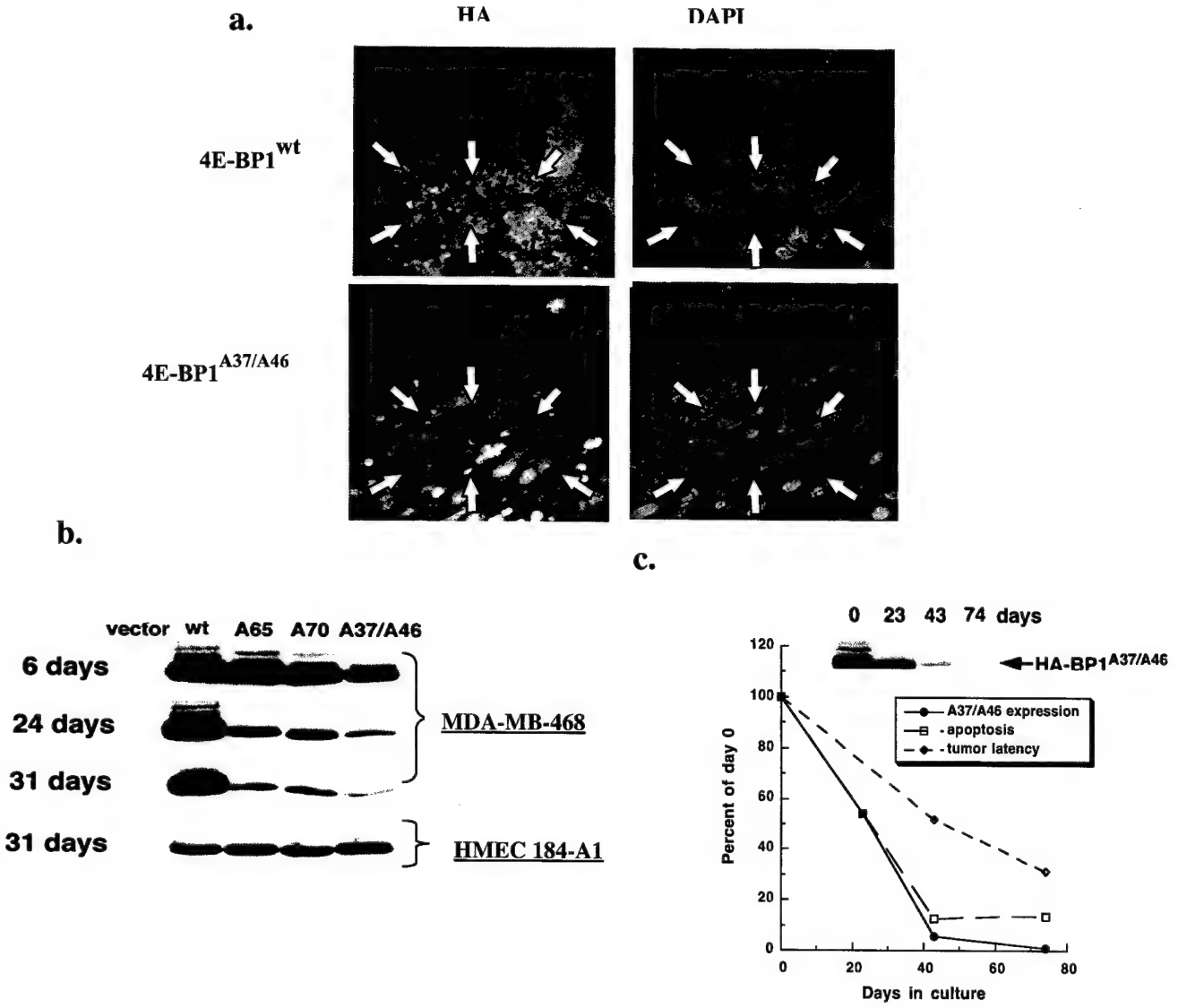


Figure 5. Loss of hypophosphorylated 4E-BP1 expression during propagation of breast cancer cells in vivo and in vitro.

a: Immunohistochemistry analysis for HA-4E-BP1 in tumors formed by HA-4E-BP1^{wt} or HA-4E-BP1$^{A37/A46}$ MDA-MB-468 cells. Sections of 21 day tumors were probed with anti-HA antibody and bound antibody were visualized with FITC conjugated secondary antibody. Following antibody specific staining, nuclei were counterstained with DAPI. Arrows indicate the areas of extensive death of cells with high levels of FITC fluorescence. **b:** Western blot for HA-4E-BP1 expression in course of in vitro cultivation of MDA-MB-468 and 184-A1 cells transduced with wild type or the phosphorylation site mutants of HA-4E-BP1. Extracts of transduced breast carcinoma cells and HMEC 184-A1 were analyzed by HA immonobloting at the indicated time intervals after transduction. **c:** Loss of ectopic 4E-BP1expression in HA-4E-BP1$^{A37/A46}$transduced breast carcinoma cells is associated with gain of resistance to apoptosis and restoration of tumorigenicity. MDA-MB-468 cells transduced with HA-4E-BP1$^{A37/A46}$ were seeded in regular growth medium, cultivated for 7 days (day 0 in the plot, above) and subcultivated weekly thereafter at a 1:10 split ratio. After the indicated interval, cells were divided into three aliquots and were: (1) examined for levels of 4E-BP1 by HA-immunoblotting; (2) subjected to flow cytometric quantification of apoptosis under serum-free conditions for 48 h; or (3) injected into the mammary fad pads of nude mice (six animals, 5 x 10^6

cells/injection) to assay tumor latency (time interval after cell inoculation when tumors become visible in at least 50% of animals). The data from three independent experiments are presented normalized to the percentage of values observed for each parameter on day 0.

ectopic 4E-BP1 - and among these, HA-4E-BP1$^{A37/A46}$ was nearly absent after 7-8 weeks of cultivation (Figures 5b and 5c). Of note, the loss of HA-4E-BP1$^{A37/A46}$ expression clearly paralleled reversion to an apoptosis sensitive phenotype in vitro, with restoration of tumorigenicity (Figure 5c). **Thus, increased activity of 4E-BP1 was not only sufficient, but also essential, for apoptosis sensitivity of cells in vitro, and for their low tumorigenicity in the murine xenograft model. These data are consistent with a scenario whereby expansion of transformed cells during breast tumorigenesis requires selection for permanent activation of eIF4F as a part of the program acquired by cancer cells to evade apoptosis.**

Summary of Data in Support of Aim 3

1. Enforced overexpression of 4E-BP1 in breast carcinoma cells decreases their tumorigenicity in nude mice xenograft by activating apoptosis. Preventing 4E-BP1 phosphorylation by mutating its phosphorylation sites increases the anti-tumor potential of 4E-BP1. These results establish proof of principle: aberrantly activated cap-dependent translation is not only a hallmark of malignant transformation and breast carcinogenesis but also an essential factor required for cancer cell viability and tumor formation.
2. Breast cancer cells tend to lose expression of the pro-apoptotic forms of 4E-BP1 both *in vitro* and *in vivo*. This loss parallels restoration of cell resistance to apoptosis and tumor regrowth. Mutating phosphorylation sites contributes to loss of 4E-BP1 in a rank-order that matches the pro-apoptotic potencies of mutant forms of 4E-BP1. We conclude that intensification of 4E-BP1 phosphorylation and other event leading to inactivation of 4E-BP1 are under strong selective pressure and contribute to tumor progression by providing cell survival advantages.

Key Research Accomplishments

• Our findings suggest that human breast cancer cell lines with diverse genetic backgrounds uniformly exhibit a distinct pattern of eIF4F activation compared to their non-transformed counterparts. Thus, activated cap-dependent translation is integral to the pathobiology of breast cancer.We also provide important insights into the strategies used by breast cancer cells to activate eIF4F: all breast carcinoma cells tested harbor increased translational factor eIF4GI and express hyperphosphorylated translational repressor 4E-BP1.

• Our results suggest that transfer of genes encoding translational factor eIF4E into non-transformed mammary epithelial cells confers crucial properties of malignancy: autonomy for those growth and survival signals that are provided by cell-to-cell adhesion/interaction molecules and resistance to apoptosis induced by intrinsic factors or anti-cancer drugs.

• Our analysis of cancer cells ectopically expressing 4E-BP1 revealed that the clonogenic expansion of breast carcinoma cells in vitro and their tumorigenicity in vivo are absolutely dependent on a function that is inhibited by increased levels of hypophosphorylated 4E-BP1. This suggests that sustained activation of the cap-dependent translational machinery is essential for expression of a malignant phenotype in breast cancer cells. Together, our data show that genesis and maintenance of a drug resistant neoplastic phenotype in mammary epithelial cells are strictly dependent upon the activation status of the eIF4F-mediated translational apparatus.

• Our findings provide the conceptual basis for a new approach to anticancer therapy: correcting aberrant translation as a complement to other strategies that target anti-apoptotic signaling in human neoplasm's. While ectopic 4E-BP1 increases apoptosis in breast cancer cells, even significant overexpression of wild type or hypophosphorylated forms of 4E-BP1 is nontoxic for non-transformed breast epithelial cells. This provides the opportunity to utilize this cancer cell-selective death pathway for anticancer therapy and to spare normal cells. Based on these data, we plan to employ novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

Reportable Outcomes

• Manuscripts, Abstracts, Presentations

Manuscript:

1. Li S, Sonenberg N, Gingras A-C, Peterson M, Avdulov S, **Polunovsky VA**, Bitterman PB. Translational control of cell fate: availability of phosphorylation sites on translational repressor 4E-BP1 governs its pro-apoptotic potency. *Mol Cell Biol,* 2002, 22: 2853-2861
2. Li S, Takasu T, Perlman D, Peterson M, Burrichter D, Avdulov S, Bitterman P, **Polunovsky V.** Translation factor eIF4E rescues cells from Myc-dependent apoptosis by inhibiting cytochrome c release. *J. Biol. Chem.* 278: 3015-3022, 2003
3. Avdulov S, Li S, Peterson M, Perlman D, Burrichter D, Sonenberg N, Yee D, Manivel JC, Bitterman P, and **Polunovsky VA,** Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells. *Cancer Cell* (In preparation).

Abstracts:

1. Shunan Li, Peterson M, Murray J, Schwarze J, Bitterman P and **Polunovsky VA** Activation of apoptosis by translational repressor 4E-BP1 depends on its phosphorylation status. ASCB Annual Meeting, December 9-13, San Francisco, 2000.
2. **Polunovsky VA,.** Avdulov S, Shunan Li, Peterson M, Gingras A-C, Sonenberg N, and Bitterman P. Translation control of malignancy: Translation repressor 4E-BP1 activates apoptosis in breast cancer cells in a manner dependent on its phosphorylation status. Abstract was selected for oral presentation at AACR Annual Meeting,, March 24-28, New Orleans, LA , 2001
3. Avdulov S, Peterson M, Li S, Sonenberg N, Bitterman n, and **Polunovsky V.** Translational control of malignancy and acquired chemoresistance: antiapoptotic function of the translational factor eIF4E in breast cancer cells. AACR Annual Meeting,, April 6-10, San-Francisco, CA, 2002
4. Svetlana A. Avdulov, Shunan Li, Mark Peterson, Nahun Sonenberg*, Peter B. Bitterman, and Vitaly A. Polunovsky. Translational control of malignancy: Antiapoptotic function of the translational complex eIF4F. Int J Mol Med, v.10, suppl.1, S79, 2002
5. Avdulov S. A., S. Li, D. Burrichter, M. Peterson, N. Sonenberg , P. B. Bitterman, and V. A. **Polunovsky** .Translational control of malignancy and chemoresistance in breast cancer cells: antiapoptotic function of the translational factor eIF4E. The 6th International Symposium "Cancer Detection and Prevention" February 9-12, Pasteur Institute, Paris, France, 2002
6. Avdulov S, Peterson M, Li S, Sonenberg N, Bitterman n, and **Polunovsky V.** Car-dependent translational control of tumorigenicity and chemoresistance in breast cancer cells. The DOD Cancer Research Program Meeting "Era of Hope", September 25-28, Orlando, Florida, 2002
7. Svetlana A. Avdulov, Shunan Li, Mark Peterson, David Burrichter, Nahum Sonenberg, Peter B. Bitterman, and Vitaly A. **Polunovsky**. Cap-dependent translational control of malignant phenotype in mammary epithelial cells. The Meeting "Translational Control" September 10-15, Cold Spring Harbor, NY, 2002

8. Avdulov S. A., S. Li, D. Burrichter, M. Peterson, N. Sonenberg , P. B. Bitterman, and V. A. **Polunovsky** . Translational Control of Cancer: Requirements for Upregulated Translational Complex eIF4F in Tumor Cell Viability and Tumorigenicity. Beatson International Cancer Conference "Cell Signalling and Cancer" July 6-9, Glasgow, Scotland, 2003

Presentations:

1. Takasu T, Bitterman PB, **Polunovsky VA.** Translation factor eIF4E rescues Myc overexpressing cells from drug-induced apoptosis through a Bcl-XL-mediated blockage of caspase-3 activation. Abstract was selected for oral presentation at Cold Spring Harbor Laboratory Meeting"Programmed Cell Death", September-October 1999, Cold Spring Harbor, NY, 1999.
2. **Polunovsky VA,** Bitterman P, Takasu T. Translation factor eIF4E operates through Bcl-X_L to suppress Myc-dependent apoptosis. Abstract was selected for oral presentation at ASCB Annual Meeting, December 11-15, Washington DC,1999
3. Avdulov SV, Li S, Peterson M, Gingras A-C, Sonenberg N, Bitterman P, **Polunovsky VA.** Translational control of acquired chemoresistance in breast cancer cells: anti-apoptotic function of the activated cap-dependent translational apparatus. International Meeting in Sardinia: "New Targets in Molecular Carcinogenesis." Alergo, Italy, September 23-26, 2001
4. **Polunovsky VA,.** Avdulov S, Shunan Li, Peterson M, Gingras A-C, Sonenberg N, and Bitterman P. Translational control of malignancy and chemoresistance in breast cancer cells: antiapoptotic function of the translational factor eIF4E. 6th International Symposium "Cancer Detection and Prevention" February 9-12, Pasteur Institute, Paris, France, 2002
5. **V. A. Polunovsky,** S. A. Avdulov, S. Li, D. Burrichter, M. Peterson, N. Sonenberg , P. B. Bitterman, Translational control of malignancy: Antiapoptotic function of the translational complex eIF4F. The 7th World Congress on Advances in Oncology, October 10-12, Hersonissos, Crete, Greece, 2002

• Patents

Bitterman PB, **Polunovsky VA**, Sonenberg N, Gingras A-C. Methods of Modulating Pro-Apoptotic and Anti-Apoptotic Pathways in Ras-transformed Cells.
NIH Invention Disclosure Number 1450401-98-0033

• Degrees obtained that are supported by this research

Not available

• Informatics such as databases and animal models, etc

Not available

• Funding applied for based on work supported by this award

Completed:

The Twin Cities Affiliate of Susan G. Komen Breast Cancer Foundation (**V. Polunovsky** PI)

The Pro-Apoptotic Function of Translational Repressor 4E-BP1 in Breast Carcinomas.

03/15/2000-03/14/2001

Active:

NIH/NCI RFA CA-00-002

Translational Apparatus as a Target for Cancer Drug Discovery (**V. Polunovsky** PI)

04/01/2001-03/31/2005 $350,000 Direct cost/year

Pending:

1. NIH/NCI U01 04/01/2004 – 03/31/2009
   Translational Activation to Model Breast Cancer in Mice (**V. Polunovsky** PI)

2. US AMRMC DOD FY03 BCRP
   Dysfunction of the 4E-BP Translational Reppressors as a Factor of Breast Cancer Predisposition

• Employment or research opportunities applied for/or received

Polunovsky V.A. (PI) Full Professor, University of Minnesota, Department of Medicine

List of personnel:

Polunovsky V.A. (PI)	Full Professor,	20%	
Van K. Michalec	Scientist	20%	
Shui Cheng Chen	Scientist	50%	10/1/99 – 4/30/00
Svetlana Chochina/Avdulov	Research Associate	20%	5/1/00 - 9/30/03

Conclusions

We **hypothesized** that the activation state of translation initiation factor eIF4E is one critical determinant of chemosensitivity in breast cancer. Our **objective** was to experimentally test the **idea** that targeted disruption of the anti-apoptotic function of eIF4E can sensitize breast carcinoma cells to therapeutic doses of a non-genotoxic cytostatic agents and/or to low concentrations of genotoxic agents.
We proposed to test our hypothesis through 3specific aims:
Aim I: *In vitro*, examine whether levels of eIF4E expression determine chemoresistance in human breast cancer cell lines.
Aim 2. *In vitro*, develop a novel protocol(s) for breast carcinoma treatment that will include pharmacological inhibitors of eIF4E activity and non-toxic doses of anti-cancer agents.
Aim 3. Utilize a nude mouse xenotransplantation preclinical model to examine proof of principle: reduction in eIF4E activity chemosensitizes human breast cancer cells.

Aim 1: Our results argue that breast cancer cells acquire a set of metabolic and regulatory lesions that aberrantly promote assembly of the eIF4F initiation complex. Our findings provide important insights into the strategies used by breast cancer cells to activate eIF4F. All breast carcinoma cells tested harbor increased eIF4GI and express hyperphosphorylated 4E-BP1.
Aim 2: Our results clearly show that enforced overexpression of translational factor eIF4E induces some aspects of transformation in immortalized human mammary epithelial cells and increases their resistance to spontaneous and drug-induced apoptosis. In line with these observations, targeted disruption of the cap-dependent translational complex by ectopic overexpression of the translational repressor 4E-BP1 stimulates apoptosis and abrogates chemoresistance in breast carcinoma cells harboring diverse oncogenic alteration in a manner dependent on a phosphorylation status of 4E-BP1. These findings confirmed proof of principle: breast cancer cells acquire metabolic alterations leading to increased cap-dependent translation to oppose transformation-related activation of their intrinsic apoptotic program and resist spontaneous and drug-induced apoptosis.
Aim 3: We demonstrated that enforced overexpression of the translational repressor 4E-BP1 in breast carcinoma cells decreases their tumorigenicity in nude mice xenograft by activating apoptosis. We also found that preventing 4E-BP1 phosphorylation by mutating its phosphorylation sites increases the anti-tumor potential of 4E-BP1. In line with these observations, we found that breast cancer cells tend to lose expression of the pro-apoptotic forms of 4E-BP1 both *in vitro* and *in vivo*. This loss parallels restoration of cell resistance to apoptosis and tumor regrowth. Thus, increased activity of 4E-BP1 is not only sufficient, but also essential, for apoptosis sensitivity of cells *in vitro*, and for their low tumorigenicity in the murine xenograft model.

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Appendices

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 June 2001 (07.06.2001)

PCT

(10) International Publication Number
WO 01/40293 A2

(51) International Patent Classification[7]: C07K 14/47

(21) International Application Number: PCT/CA00/01465

(22) International Filing Date: 1 December 2000 (01.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/168,398 2 December 1999 (02.12.1999) US

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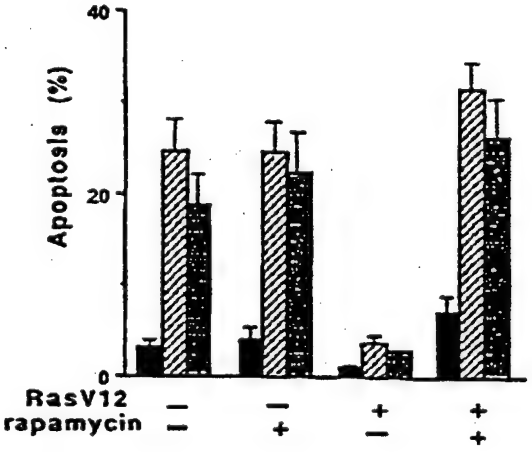
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

[Continued on next page]

(54) Title: METHOD OF MODULATING PROAPOPTOTIC AND ANTIAPOPTOTIC PATHWAYS IN CELLS



(57) Abstract: More than 30 % of human malignancies harbor encogenic Ras. Both pro-apoptotic and anti-apoptotic pathways emanate from encogenic Ras with survival being dominant. Ras survival signaling is thought to be controlled by transcriptional and post-translational processes. The present invention shows that a repressor of cap-dependent translation initiation, 4E-BP1, selectively activates apoptosis in Ras-transformed fibroblasts and eliminates Ras-induced chemoresistance. These effects of 4E-BP1 are strictly dependent on its ability to sequester translation initiation factor eIF4E, thereby preventing its assembly into an active pre-initiation complex. These results suggest that translational control is critical for prevention of apoptosis and resistance to antitumor agents in Ras-transformed cells.

WO 01/40293 A2

MOLECULAR AND CELLULAR BIOLOGY, Apr. 2002, p. 2853–2861 Vol. 22, No. 8
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.8.2853–2861.2002

Translational Control of Cell Fate: Availability of Phosphorylation Sites on Translational Repressor 4E-BP1 Governs Its Proapoptotic Potency

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Received 17 September 2001/Returned for modification 6 November 2001/Accepted 14 January 2002

Translational control has been recently added to well-recognized genomic, transcriptional, and posttranslational mechanisms regulating apoptosis. We previously found that overexpressed eukaryotic initiation factor 4E (eIF4E) rescues cells from apoptosis, while ectopic expression of wild-type eIF4E-binding protein 1 (4E-BP1), the most abundant member of the 4E-BP family of eIF4E repressor proteins, activates apoptosis—but only in transformed cells. To test the possibility that nontransformed cells require less cap-dependent translation to suppress apoptosis than do their transformed counterparts, we intensified the level of translational repression in nontransformed fibroblasts. Here, we show that inhibition of 4E-BP1 phosphorylation by rapamycin triggers apoptosis in cells ectopically expressing wild-type 4E-BP1 and that expression of 4E-BP1 phosphorylation site mutants potently activates apoptosis in a phosphorylation site-specific manner. In general, proapoptotic potency paralleled repression of cap-dependent translation. However, this relationship was not a simple monotone. As repression of cap-dependent translation intensified, apoptosis increased to a maximum value. Further repression resulted in less apoptosis—a state associated with activation of translation through internal ribosomal entry sites. These findings show: that phosphorylation events govern the proapoptotic potency of 4E-BP1, that 4E-BP1 is proapoptotic in normal as well as transformed fibroblasts, and that malignant transformation is associated with a higher requirement for cap-dependent translation to inhibit apoptosis. Our results suggest that 4E-BP1-mediated control of apoptosis occurs through qualitative rather than quantitative changes in protein synthesis, mediated by a dynamic interplay between cap-dependent and cap-independent processes.

Apoptosis in higher eukaryotes is controlled by a precisely orchestrated interaction among regulatory and effector molecules that function to eliminate unwanted cells during development, an immune response, tissue repair, and oncogenesis. Control points for apoptosis have been identified at different levels of biological regulation, including translational control. The efficiency of utilization of mRNA encoding a number of potent positive and negative regulators of apoptosis is tightly regulated (reviewed in references 4, 5, and 16).

Translational control is exerted predominantly by regulating the quantity, activity and integrity of the cap-dependent translation initiation apparatus (reviewed in references 15, 31, 32, and 33). In mammals, a trimolecular complex designated eukaryotic initiation factor 4F (eIF4F) initiates cap-dependent translation. It consists of three proteins: eIF4E, which binds the 7-methyl guanosine cap at the 5′ mRNA terminus; eIF4A, an mRNA helicase; and an eIF4G family member (eIF4GI or eIF4GII), which serves as a docking protein binding eIF4E and eIF4A. eIF4G also binds the adapter protein eIF3 which targets the intact eIF4F complex to the 40S subunit of the ribosome. Translation can also be initiated in a cap-independent fashion through internal ribosomal entry sites (IRES) (reviewed in references 3, 18and 27). When this occurs, sequences in the 5′-untranslated region of mRNA are capable of directly binding to domains on eIF4G family proteins allowing cap-independent recruitment of the transcript to the 40S ribosomal subunit.

The translational function of eIF4E is negatively regulated by a family of the eIF4E-binding proteins (4E-BPs), which share a motif with eIF4G allowing them to bind and sequester eIF4E in a competitive manner 19, 20, 26; reviewed in references 32 and 33). In this regard, the affinity of the prototype family member 4E-BP1 (also known as PHAS-I) for eIF4E is regulated by the phosphorylation state of six serine/threonine (S/T) residues on the 4E-BP1 molecule (T37, T46, S65, T70, S83, and S112, numbered according to the human 4E-BP1 sequence) (8, 38). In response to exogenous stimuli, such as growth factors and insulin, 4E-BP1 appears to be phosphorylated in a stepwise fashion, with phosphorylation of the two amino-terminal threonines (T37 and T46) functioning together to promote subsequent phosphorylation of the carboxy-terminal sites (9, 10, 24, 25). In addition, phosphorylation at T70 facilitates phosphorylation at S65 (25). Growth factors and hormones signal phosphorylation of 4E-BPs through the Akt/protein kinase B kinase cascade that includes FRAP/mTOR (FKBP12–rapamycin-associated protein or mammalian target of rapamycin) (reviewed in references 12 and 34). FRAP/mTOR mediates phosphorylation of 4E-BP1 at residues T37 and T46 and also impacts phosphorylation of S65 and T70. In

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general, accumulation of phosphate residues on 4E-BP1 decreases its affinity for eIF4E, which becomes free to bind eIF4G and initiate translation (9, 10, 24, 25).

Key components of the translation initiation apparatus have been directly implicated as targets and regulators of apoptosis (reviewed in references 2, 5, and 17). For some time it has been recognized that inhibition of protein synthesis is an early biochemical event in the process of apoptosis (7, 23, 39). At the onset of apoptosis, eIF4G is cleaved by activated caspases and cap-dependent translation abruptly decreases (5, 21). Although it is established that translation of mRNA in a cap-dependent manner is suppressed during apoptosis, this is not the case for translation of transcripts bearing an IRES, which may actually be increased (13, 17). Several studies have identified truncated forms of eIF4G family members that can support protein synthesis in apoptotic cells through an IRES. One eIF4GI cleavage product, an M-FAG (for middle fragment of apoptotic cleavage of eIF4G fragment), contains binding sites for eIF4E and eIF4A and supports some cap-dependent initiation as well as initiation utilizing an IRES (5). It shares 32% homology with a member of the eIF4G protein family, p97/DAP5/NAT1, which lacks the N-terminal eIF4E-binding site and is therefore unable to initiate cap-dependent translation. Like the M-FAG fragment, p97/DAP5/NAT1 can sustain translation of mRNAs containing IRES, including its own mRNA (reviewed in references 9, 11, and 17). Of note, the transcript for p97/DAP5/NAT1 has an IRES, setting up a positive feedback loop as apoptosis eventuates to ensure continued translation of certain proapoptotic IRES-bearing transcripts (13). In fact, p97/DAP5/NAT1 is a potent agonist of apoptosis, and the transcripts for other proapoptotic effectors such as c-Myc and Apaf-1 contain an IRES (17). These data support the notion that among the mRNAs encoding apoptotic regulatory proteins, those suppressing apoptosis are cap dependent, whereas those translated through IRES support the execution of apoptosis (13, 28). In accord with this formulation, ectopic expression of eIF4E rescues cells from apoptosis induced by growth factor restriction or cytostatic drugs (29, 35), while ectopic expression of wild-type 4E-BP1 activates apoptosis in Ras-transformed fibroblasts, leaving nontransformed cells viable (28).

While this body of evidence highlights the importance of translational control in apoptosis, the difference in viability between transformed and nontransformed cells when 4E-BP1 is ectopically expressed remains unexplained. Even a 20-fold increase in 4E-BP1 expression in nontransformed fibroblasts does not activate apoptosis (28). This finding raises the question of whether nontransformed cells are more able than their transformed counterparts to maintain the critical balance of antiapoptotic and proapoptotic proteins needed for viability in the face of increased levels of 4E-BP1 or whether the downstream repertoire of apoptotic regulatory events is fundamentally different after malignant transformation. Therefore, our objective in the present study was to determine whether intensifying repression of cap-dependent initiation by dephosphorylation of 4E-BP1 triggered apoptosis in nontransformed fibroblasts. We employed two complementary approaches: (i) pharmacological blockade of ectopic and endogenous 4E-BP1 phosphorylation with rapamycin and (ii) ectopic expression of 4E-BP1 mutants lacking specific phosphorylation sites. Here, we show that inhibition of 4E-BP1 phosphorylation by rapamycin triggers apoptosis in nontransformed fibroblasts ectopically expressing wild-type 4E-BP1. We also demonstrate that transient or stable expression of phosphorylation site mutants in nontransformed fibroblasts potently activates apoptosis in a phosphorylation-site specific manner but not in strict accord with the mutant forms' ability to repress cap-dependent translation.

MATERIALS AND METHODS

4E-BP1 expression vectors. Single and double 4E-BP1 phosphorylation mutants were generated by using PCR site-directed mutagenesis of human 4E-BP1 sequences cloned into the cytomegalovirus-based pACTAG vector, as described (9, 28). For stable transfection, the original (wild-type) and mutant 4E-BP1 sequences were inserted into the mammalian expression vector pSR *puro* between *Eco*RI and *Bam*HI sites (pSR *puro*/4E-BP1 vector). For transient transfection and clonogenic assays, the wild-type and mutant 4E-BP1 sequences were subcloned into the pACTAG-2 construct containing an amino-terminal three-hemagglutinin (three-HA) tag and a neomycin resistance gene cassette. The resulting vectors (pACTAG *neo*/HA-4E-BP1) were sequenced in their entirety, and the coding regions were found to be free of undesired mutations. To evaluate rates of cap-dependent translation and translation mediated by an IRES, we used a bicistronic reporter plasmid, pcDNA3-rLuc-polIRES-fLuc (28), modified for expression in eukaryotic cells by insertion of the pCMV promoter.

Cell culture, transfection, and clonogenic assay. Cloned rat embryo fibroblasts (CREF) were described previously (28). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS). For both stable and transient transfection, 3.5×10^5 CREF were plated in 35-mm wells of six-well clusters. After 24 h, cells were transfected with 4E-BP1 expression plasmids or with an empty vector using Lipofectamine Plus reagent (Gibco) according to the manufacturer's instructions. To generate clonal cell lines constitutively expressing the wild type or serine-to-alanine (A) mutants of 4E-BP1 at amino acid 65, CREF were transfected with the pSR *puro*/4E-BP1 vector and after 2 days were selected for resistance to 1-μg/ml puromycin (Sigma) for a period of 2 weeks. Individual puromycin-resistant clonal lines were screened for expression of 4E-BP1. For transient gene transfer, cells were transfected with the empty pACTAG *neo*/HA vector or with the pACTAG *neo*/HA-4E-BP1 expression plasmids encoding either wild-type or mutant 4E-BP1. Floating and adherent cells were collected after 48 h and subjected to immunoblotting or flow cytometric assays to detect expression of HA and 4E-BP1 and to quantify apoptotic frequency. Transfection efficiency was quantified by flow cytometric analysis of the percentage of HA-positive cells after incubation of transfected cells with primary HA antibody, followed by staining with fluorescein-conjugated secondary antibody, as described (28). Values for all constructs clustered around 23% (range, 20.3 to 24.9%).

Apoptosis assay. To quantify apoptotic frequency, cells were washed in ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol, followed by incubation with propidium iodide stain mixture (50 μg of propidium iodide/ml, 0.1% Triton X-100, 37 μg of EDTA/ml, 2.5 U of RNase/ml in PBS) for 60 min at room temperature. The percentage of cells with hypodiploid DNA content was determined with a FACScan flow cytometer (Becton Dickinson) with the CellQuest program.

Immunoblot analysis. Floating and adherent cells were collected, washed with cold PBS, and lysed by three successive freeze-thaw cycles in 50 mM Tris, (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg of pepstatin A/ml, 10 mM sodium pyrophosphate, 50 mM β-glycerolphosphate, and 0.1 mM Na_3VO_4. Cellular proteins (50 μg) were resolved by either an 8 to 15% linear gradient or sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Blots were blocked in TBST with 5% dry milk and 0.05% Tween-20. To detect 4E-BP1, eIF4G1 and eIF4E blots were incubated with antibody, washed, incubated with the appropriate horseradish peroxidase-coupled secondary antibody, and detected by enhanced chemiluminescence as described (28).

Cap affinity binding assay. m^7GTP-Sepharose chromatography was performed as described previously (28) by applying 250 μg of cell lysate protein in a volume of 250 μl to 20 μl of packed m^7GTP-Sepharose beads. Captured proteins were eluted with buffer containing 70 μM m^7GTP and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to transfer and immunoblot analysis.

Quantification of cap-dependent and IRES-mediated translation. Cells were cotransfected with 1 μg of pcDNA3-rLuc-polio-fLuc and either wild-type or mutant pACTAG/HA-4E-BP1 (1 μg) or with 1 μg of pACTAG/HA vector. Cells were rinsed with PBS 24 h after transfection and incubated with the passive lysis buffer (Promega) for 15 min. Cell debris was pelleted by centrifugation, and triplicate supernatant samples were assayed for *Renilla* and firefly luciferase activities in a Lumat LB 9507 luminometer (BG&G, Berthold, Germany) using the Promega dual luciferase reporter system.

Statistics. Statistical analyses were performed using one-way analysis of variance with Dunnett's multiple comparison test (S-PLUS Guide to Statistical and Mathematical Analysis, version 4.0; Insightful, Seattle, Wash.). A P value of <0.05 was considered significant.

RESULTS

Rapamycin and ectopic 4E-BP1 synergistically impair assembly of the cap-dependent initiation complex and activate apoptosis. When wild-type 4E-BP1 is ectopically expressed in nontransformed fibroblasts (CREF), it is unable to activate apoptosis (28). The phosphorylation status of 4E-BP1 modulates its ability to sequester eIF4E and repress cap-dependent translation, with hypophosphorylated forms associating most avidly. To determine if decreasing the phosphorylation status of ectopically expressed 4E-BP1 increased its proapoptotic potency, we developed clonal cell lines of CREF ectopically expressing wild-type 4E-BP1 at different levels and manipulated 4E-BP1 phosphorylation status pharmacologically. Phosphorylation of 4E-BP1 occurs in a FRAP/mTOR kinase-dependent manner, a reaction inhibited in vivo by rapamycin (1, 10, 12, 34). We selected clonal fibroblast lines expressing 4E-BP1 at high or intermediate levels and compared the viability of each to untransfected cells in the presence or absence of rapamycin (Fig. 1). To minimize the effect of extracellular signaling, this set of experiments was conducted in medium containing 0.1% FCS, a condition in which endogenous 4E-BP1 expression is barely detectable.

As expected, addition of rapamycin decreased expression of hyperphosphorylated 4E-BP1 γ and increased the expression of hypophosphorylated 4E-BP1 α (Fig. 1A). The integrity of the eIF4F cap-dependent translation initiation complex was examined using cap analogue capture of eIF4E and its binding partners in cell lysates, followed by immunoblot analysis. This analysis revealed no change in eIF4E partnering after rapamycin treatment of nontransfected cells; eIF4GI remained the predominant binding partner of eIF4E (Fig. 1B). In the clonal line expressing intermediate levels of 4E-BP1, there was no detectable decrease in the binding of eIF4G1 to eIF4E. Rapamycin inhibited phosphorylation of 4E-BP1 and displaced eIF4GI from eIF4E, indicating disassembly ofeIF4F. In the high-level expressors, where 4E-BP1 predominated as the binding partner of eIF4E, rapamycin caused a discernible bias toward hypophosphorylated 4E-BP1 bound to the cap-captured eIF4E. Of note, in association with these biochemical changes, rapamycin significantly increased the frequency of apoptosis in cells ectopically overexpressing 4E-BP1 (Fig. 1C and D). In accord with our previously published findings, ectopic expression of 4E-BP1 in the absence of rapamycin did not trigger apoptosis. Thus, pharmacological inhibition of 4E-BP1 phosphorylation activates apoptosis in nontransformed fibroblasts ectopically expressing wild-type 4E-BP1.

Mutating Ser-65 to Ala increases the proapoptotic activity of ectopically expressed 4E-BP1. To examine the relationship between the expression level of hypophosphorylated 4E-BP1 and susceptibility to apoptosis, we developed a series of clonal cell lines stably expressing a serine-to-alanine mutation of 4E-BP1 at residue 65 (designated 4E-BP1A65). We selected this residue, since phosphorylation of Ser-65 is rapamycin sensitive and has the least impact on phosphorylation of other S/T residues in the 4E-BP1 molecule (8, 9, 10, 25). Four clonal cell lines of A65 were chosen that represented a 60-fold range of expression, and displayed mostly hypophosphorylated α and β forms (Fig. 2A). Apoptosis was quantified under standard culture conditions after serum withdrawal and after exposure to either genotoxic (e.g., camptothecin) or nongenotoxic (e.g., lovastatin) cytostatic agents. While the magnitude of the apoptotic response differed among the stimuli examined (with serum withdrawal exerting the most potent proapoptotic effect, followed by lovastatin and camptothecin), there was a reproducible rank order of susceptibility to apoptosis that exactly paralleled the level of ectopic 4E-BP1A65 expression (Fig. 2B). Apoptotic frequency ranged from 2 to 18% for the mock-transfected line, peaking at 17 to 44% for the highest expressor examined. Since even highly overexpressed wild-type 4E-BP1 does not activate apoptosis in this cell system, these data suggest that mutating Ser-65 to alanine is sufficient to convert 4E-BP1 from a nonapoptotic to a proapoptotic form for CREF. They also establish a direct relationship between the level of mutant 4E-BP1 expressed and fibroblast susceptibility to apoptosis.

To examine the impact of increasing cellular levels of 4E-BP1A65 in the absence of clonal selection, fibroblasts were transiently transfected with expression vectors that included HA epitope-tagged wild-type or 4E-BP1A65 sequences. Both HA expression and DNA content were quantified by using a two-parameter flow cytometric analysis. Analysis of nontransfected fibroblasts revealed that the basal frequency of apoptosis (hypodiploid DNA content) was approximately 3% (2.4% in the example shown in Fig. 2C) and permitted calibration of the background HA signal in the apoptotic cell population (mean signal intensity = 7.3, expressed in arbitrary units). Expression of HA vector increased the frequency of apoptosis to 6.9% and, as expected, increased the HA signal intensity in all cells analyzed, independent of DNA content. Introduction of wild-type 4E-BP1 into fibroblasts yielded an apoptotic frequency similar to that seen with empty vector. There was, however, a significant skew of hypodiploid cells toward HA positivity (mean signal intensity = 36.8), indicating increased DNA degradation in cells harboring exogenous 4E-BP1. Transfection with the A65 mutant doubled the frequency of apoptotic cells, with the vast majority being HA bright (mean signal intensity = 35.9). Of note, the HA-bearing constructs resulted in a very narrow range of HA positivity (20.7 to 24.4%), suggesting that differences in transfection efficiency did not account for the results observed. These data confirm the proapoptotic activity of 4E-BP1A65 in nontransformed fibroblasts.

Regulation of cell viability by 4E-BP1 is phosphorylation site specific. To comprehensively examine the impact of 4E-BP1 phosphorylation state on fibroblast growth, we set out to quantify the colony-forming efficiency of fibroblasts transfected

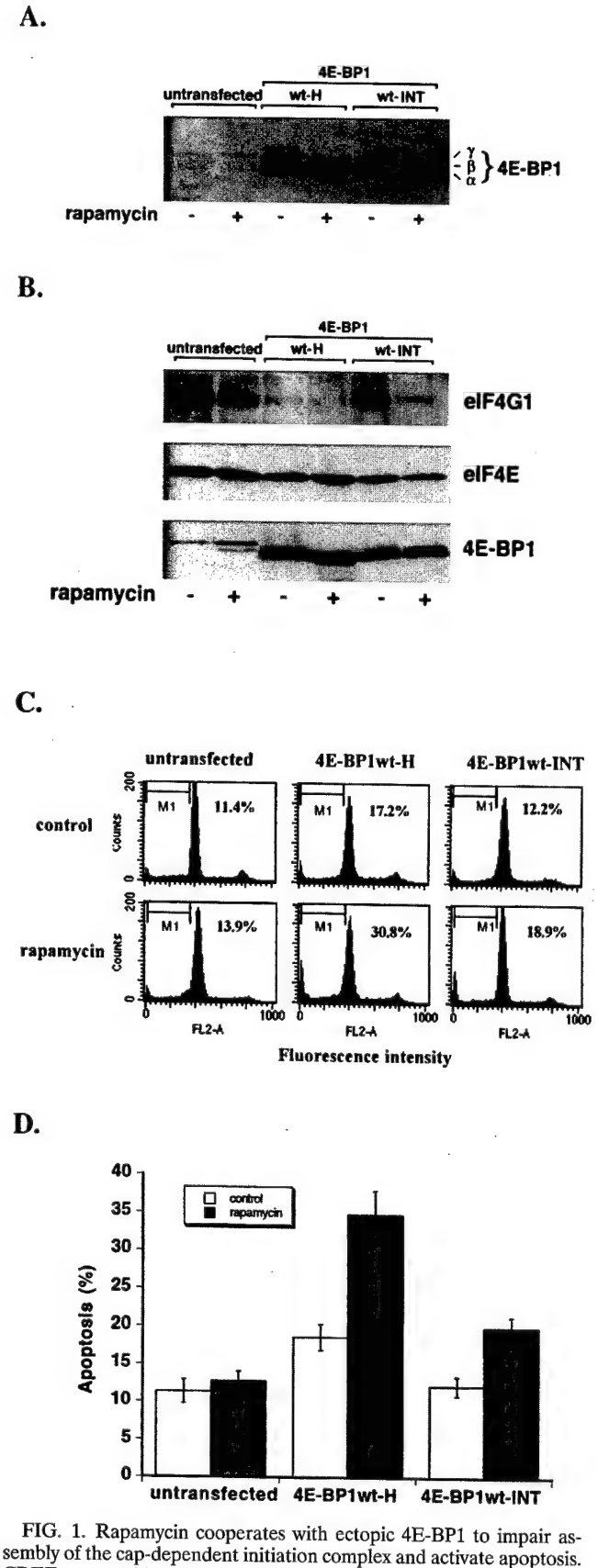

FIG. 1. Rapamycin cooperates with ectopic 4E-BP1 to impair assembly of the cap-dependent initiation complex and activate apoptosis. CREF were stably transfected with untagged wild-type (wt) 4E-BP1. Eight neomycin-resistant clones were developed and analyzed by immunoblotting, and two clonal cell lines were expanded, one expressing high (wt-H) and one expressing intermediate (wt-INT) levels of ectopic 4E-BP1. Nontransfected CREF and cells ectopically expressing 4E-BP1 were preincubated with DMEM plus 0.1% FCS for 18 h, and cultures were continued in the presence or absence of 75 nM rapamycin for 6 (A and B) or for 24 (C and D) h. (A) Immunoblot analyses of steady-state 4E-BP1 expression. (B) Immunoblot analysis of 4E-BP1 and eIF4G1 associated with eIF4E bound to m^7GTP-Sepharose. (C) Flow cytometric data from a representative experiment. The percent hypodiploid DNA is indicated in the upper right corner of each plot. (D) Apoptotic frequency (hypodiploid DNA content determined by flow cytometry). The mean ± standard deviation (SD) of three independent experiments is shown.

with a neomycin resistance cassette bearing sequences for wild-type 4E-BP1 or S/T-to-Ala mutants at the phosphorylation sites indicated (Fig. 3A). We employed three mutant forms of 4E-BP1: one double mutant (A37/A46) to eliminate phosphorylation N terminal to the eIF4E binding site, and two single mutants, A65 and A70, at phosphorylation sites C terminal to the eIF4E binding site. To detect any potential experimental bias resulting from systematic differences in ectopic wild-type or mutant 4E-BP1 expression due to self repression (i.e., negative feedback from the ectopic protein itself leading to decreased translation of ectopic and/or endogenous 4E-BP1 despite identical gene transfer efficiency), cells were transiently transfected in parallel with a pACTAG *neo* vector encoding wild-type or mutant HA-tagged 4E-BP1 using identical procedures in four independent experiments. Immunoblot analysis of 4E-BP1 expression showed equivalency for endogenous 4E-BP1 and all forms of HA-tagged 4E-BP1, except for cells transfected with A37/A46. For this double mutant, endogenous and exogenous 4E-BP1 was reproducibly expressed at a lower level (Fig. 3B). Replication of this experiment employing a retroviral gene transfer procedure led to similar results (not shown). To detect any experimental bias due to systematic differences in gene transfer efficiency, flow cytometric analysis was carried out. The proportion of cells expressing each mutant form (i.e., HA bright) was similar, ranging from 20.3 to 24.9% (not shown). These data indicated that self-repression, not differences in the proportion of transfected cells, most likely accounted for decreased expression of the A37/A46 mutant.

After transfection of CREF with empty vector, the wild type, and each mutant form, cultures were continued for 2 weeks and colony-forming efficiency was scored as the number of G418-resistant colonies produced by fibroblasts transfected by each HA-tagged 4E-BP1 vector, normalized to colony counts after transfection with empty HA vector. Ectopic expression of wild-type 4E-BP1 did not significantly alter CREF colony formation (Fig. 4A and B), a result in accord with its lack of impact on apoptosis in nontransformed fibroblasts (28). In sharp contrast, colony formation by fibroblasts transfected with S/T phosphorylation mutants was significantly reduced, ranging from 20% fewer colonies in response to transfer of the A37/A46 mutant to an 80% reduction in colony number after transfer of the A70 mutant. Values for A65 were intermediate.

The reduction of colony formation observed could have resulted from inhibition of cell cycle transit, activation of cell death, or both. We explored these possibilities by subjecting

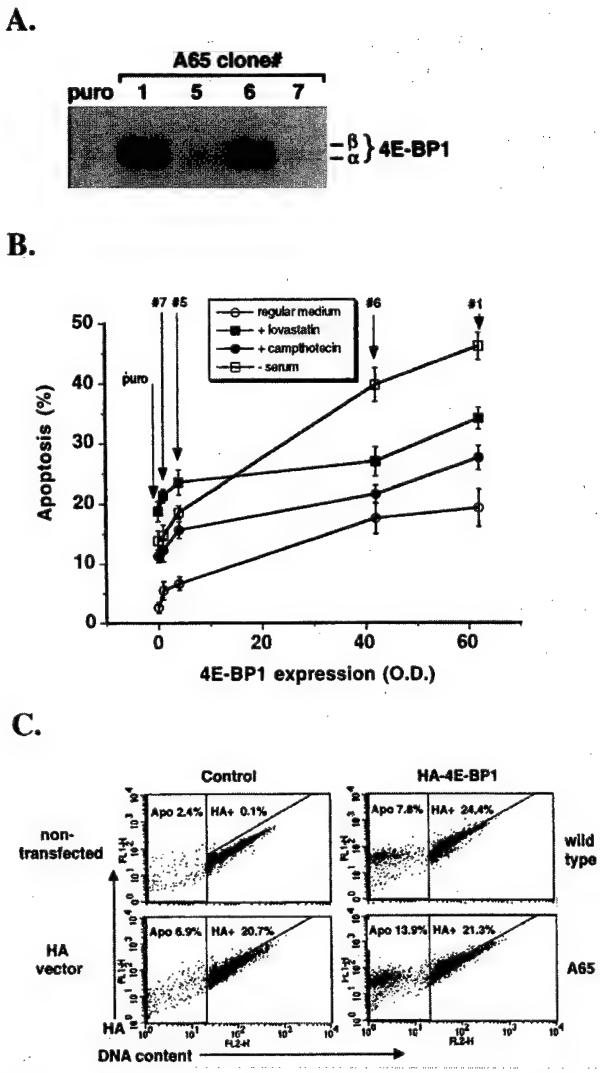

FIG. 2. Activation of apoptosis by 4E-BP1A65 depends on its level of expression. CREF were stably transfected with a construct encoding an untagged A65 mutant of 4E-BP1. Eleven neomycin-resistant clones were isolated, and expression of 4E-BP1 was assessed by immunoblotting. Four clonal cell lines expressing a range of ectopic 4E-BP1A65 were propagated. (A) Immunoblot analysis of 4E-BP1 expression in A65 clones 1, 5, 6, and 7 compared to a clonal line bearing an empty puromycin vector (puro). (B) Apoptosis assay. Cells were cultured for 48 h in serum-free medium or incubated for 24 h in growth medium (DMEM plus 10% FCS) with or without 5 μM lovastatin or 300 nM camptothecin. Cells were fixed and stained with propidium iodide, and the percentage of cells with hypodiploid DNA content was quantified by flow cytometry. The results shown represent the mean ± SD of three independent experiments. (C) Two-parameter flow cytometric analysis of HA–4E-BP1 expression and apoptosis. CREF were transiently transfected with pACTAG encoding the indicated form of HA–4E-BP1 or with empty pACTAG. Cells were fixed with 1% formaldehyde, postfixed with 70% ethanol, exposed to fluorescein-conjugated anti-HA antibody (5 μg/ml), and stained with propidium iodide. HA expression (green fluorescence) (vertical axis) and DNA content (red fluorescence) (horizontal axis) were quantified in controls (nontransfected, upper left; empty HA vector, lower left) and after transfection with either HA–4E-BP1wt (upper right) or the A65 mutant (lower right). Gating parameters are as follows: the diagonal line in each panel defines the boundary between HA-positive and -negative cells, and the vertical line in each panel defines the hypodiploid DNA boundary. The result of a representative experiment is shown (three independent experiments yielded similar results).

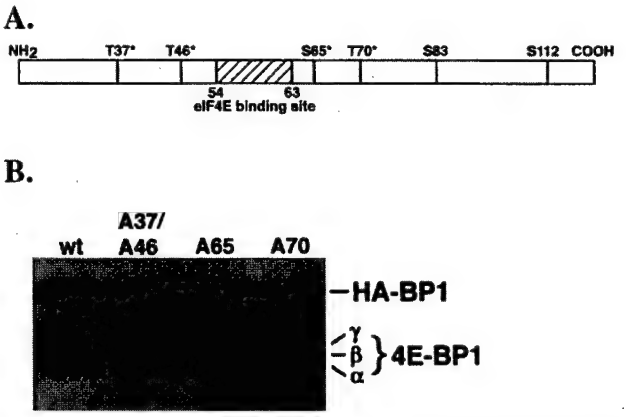

FIG. 3. Steady-state levels of endogenous and ectopic 4E-BP1 after transfection with wild-type or mutant forms of 4E-BP1. (A) Shown are the positions of the four serine/threonine phosphorylation sites of 4E-BP1 mutated to alanine relative to the eIF4E binding site (designated with asterisks), and serines 83 and 112, which were left nonmutated in our studies. (B) Immunoblot assessment of 4E-BP1 expression. CREF were transfected with 1 μg of empty pACTAG or with 1 μg of pACTAG encoding either wild-type 4E-BP1 (wt), or one of indicated S/T-to-alanine 4E-BP1 mutants (all HA epitope tagged). Cells were analyzed for steady-state expression of ectopic (HA-BP1) and endogenous (phosphorylation forms α, β, and γ are shown) 4E-BP1. Presented is a blot representative of four independent transfection experiments.

CREF to flow cytometric analysis after transient transfection with constructs encoding wild-type or mutant HA-BP1. The proportion of cycling cells (S phase plus G_2/M) was not significantly altered by any of the 4E-BP1 constructs (Fig. 4C), whereas the proportion of cells with hypodiploid DNA content (apoptotic cells) was increased from 1.7- to 2.5-fold by the phosphorylation site mutants but not by the wild-type 4E-BP1. Among wild-type and mutant forms, the rank order of potency in activating DNA degradation generally paralleled the potency in suppressing colony formation with A70 > A65 > A37/A46 > wild type. These data suggest that the 4E-BP1 phosphorylation site mutants suppressed CREF colony formation by activating apoptosis and not by blocking cell cycle transit. However, these results leave open the question of whether the relatively low potency of the A37/A46 mutant was a true reflection of its decreased intrinsic ability to modulate cell fate or simply resulted from insufficient expression.

Relationships among cap-dependent translation, translation mediated by an IRES, and fibroblast viability. We next investigated whether the rank order of proapoptotic potency of each 4E-BP1 phosphorylation site mutant matched its potency in repressing cap-dependent protein synthesis. 4E-BP1-induced alterations of cap-dependent and IRES-mediated translation were detected by cotransfecting fibroblasts with wild-type HA–4E-BP1 or mutant constructs, along with a bicistronic reporter vector encoding *Renilla* and firefly luciferases. In this system, translation of *Renilla* luciferase was cap dependent, whereas translation of firefly luciferase was mediated by an IRES (30).

Expression of either wild-type or mutant 4E-BP1 suppressed cap-dependent translation with a rank order of inhibitory potency conforming to previously published results (25): A37/A46 > A70 > A65 > wild type (Fig. 5A). In this regard, the

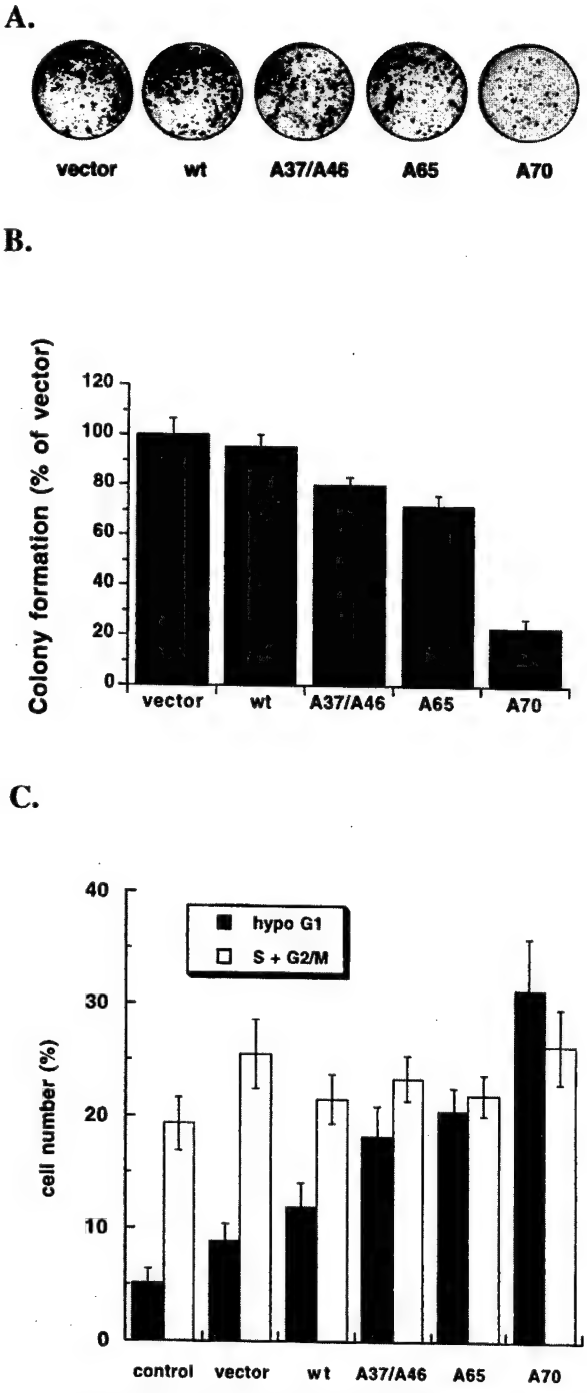

FIG. 4. Mutating 4E-BP1 phosphorylation sites reduce fibroblast colony formation and increase apoptosis. CREF were transfected either with vector, the 4E-BP1 wild type (wt), or mutant forms of 4E-BP1. (A and B) Transfected cells were seeded at low density and cultured for 2 weeks. G418-resistant colonies were scored after fixation with 10% formaldehyde and Coomassie staining. Shown is a photograph of a representative set of dishes (A) and colony counts (B), expressed as the ratio of the number of colonies formed by cells transfected with the indicated 4E-BP1 to the number of colonies formed by cells transfected with empty pACTAG vector (results represent the mean ± SD of three independent experiments). (C) DNA content after ectopic expression of wild-type or mutant 4E-BP1. After the transfection procedure, cells were fixed with 70% ice-cold ethanol and stained with propidium iodide, and the frequency distribution of red fluorescence was defined. Shown is the proportion of nontransfected and transfected cells exhibiting hypodiploid DNA content (hypo G_1), taken as an index of apoptosis, or hyperdiploid DNA content (S + G_2/M), taken as a measure of proliferation.

viability index [(1 − % hypodiploid 4E-BP1 transfected cells)/(1 − % hypodiploid vector transfected)] followed cap-dependent translational repression for the A70, A65, and wild-type forms of 4E-BP1 (Fig. 5B), which left IRES-mediated translation unaltered. In contrast, while A37/A46 clearly exerted the most potent repression of cap-dependent translation despite a lower level of ectopic protein expression (70% inhibition compared to vector control), it was the least potent of the tested mutants as an activator of apoptosis and significantly stimulated translation via the IRES. Thus, our data indicate that the relationship between 4E-BP1-induced inhibition of cap-dependent translation and reduction of cell viability is not monotonic.

To distinguish an effect resulting from mutating residues 37 and 46 from one due to the alanine substitutions per se, we studied colony-forming efficiency, apoptosis, and translational repression of fibroblasts ectopically expressing 4E-BP1 E37/E46, in which T37 and T46 were mutated to glutamine. We found that ectopic expression of this mutant form gave results very similar to its alanine-substituted counterpart, although it was slightly less active in suppressing colony formation, activating apoptosis, suppressing cap-dependent translation, and activating translation via an IRES (not shown). Therefore, our data indicate that the proapoptotic effects observed were due to elimination of the N-terminal phosphorylation sites and their resultant impact on translation initiation.

DISCUSSION

Cell fate is subject to translational control (2, 4, 6, 16, 17), a principle that is underscored by the wide variety of human tumors and cancer cell lines in which the cap-dependent initiation apparatus is activated (reviewed in references 6 and 16). In prior studies, overexpressed eIF4E was found to prevent the death of both nontransformed and transformed fibroblasts, whereas wild-type 4E-BP1 promoted apoptosis only in transformed cells, sparing nontransformed fibroblasts (28, 29). This raised the question of whether malignant transformation fundamentally alters translational control of apoptosis or whether it serves to increase the cellular requirement for cap-dependent translation. Here, we show that nontransformed fibroblasts undergo apoptosis when repression of cap-dependent translation is intensified. Transfer of wild-type 4E-BP1 into nontransformed fibroblasts together with pharmacological blockade of 4E-BP1 phosphorylation using the FRAP/mTOR kinase inhibitor rapamycin activated apoptosis. Mutation of 4E-BP1 at residues T37, T46, S65, or T70 eliminates phosphorylation sites, resulting in mutant proteins that are able to evade physiological mechanisms of deactivation. Transfer of these mutant forms into nontransformed fibroblasts markedly decreased their ability to form colonies and increased apoptosis in a manner dependent on the presence of specific phosphorylation sites. In general, for the wild type and each mutant 4E-BP1 protein studied, we found that proapoptotic potency paralleled its ability to repress cap-dependent translation. Sur-

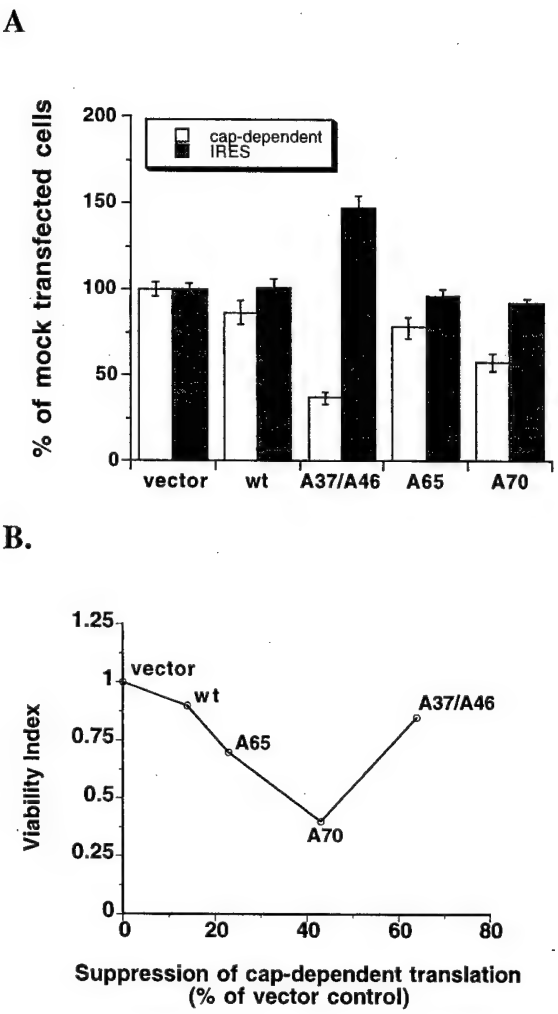

FIG. 5. Relationships among cap-dependent translation, translation mediated by an IRES, and fibroblast viability. Cells were cotransfected with pACTAG encoding wild-type or mutant HA–4E-BP1 and the pcDNA3-rLuc-polio-fLuc plasmid. (A) Cap-dependent and IRES-directed luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). The mean ± SD from three independent experiments is shown. (B) Viability as a function of cap-dependent translation. The viability index was calculated from flow cytometric data as follows: (1 − % hypodiploid 4E-BP1 transfected cells)/(1 − % hypodiploid vector transfected cells). Mean values from three independent experiments are presented.

prisingly, our data did not reveal a strict monotonic relationship between cap-dependent translation and apoptosis. Instead, we found that as repression of cap-dependent translation intensified, apoptosis increased to a maximum value (observed with the A70 mutant) and that further repression (achieved with A37/A46) resulted in less apoptosis—a state associated with activation of translation through an IRES. Together with our previous observations (28), these findings reveal that 4E-BP1 is proapoptotic in both normal and transformed fibroblasts and suggest that oncogenic transformation may confer cells with a markedly reduced capacity to block apoptosis when cap-dependent translation is suppressed by 4E-BP1. Our results also suggest that 4E-BP1-mediated control of apoptosis occurs through qualitative rather than quantitative changes in protein synthesis, mediated by a dynamic interplay between cap-dependent and cap-independent processes.

Excluding the limiting case where an insufficient quantity of structural proteins or critical enzymes is synthesized to sustain life, the role of translational control in the regulation of apoptosis is just coming into focus. In some cell types including mammalian neurons (7, 22) and fibroblasts (28), pharmacological inhibition of peptide chain elongation suppresses programmed cell death. In contrast, specific inhibition of cap-dependent translation initiation by ectopic expression of 4E-BP1 stimulates apoptosis in Ras-transformed fibroblasts in a manner dependent upon its ability to sequester eIF4E (28). Consistent with this, ectopic expression of eIF4E rescues fibroblasts from apoptotic death (29). Here, we add to the data connecting the cap-dependent initiation apparatus to the regulation of apoptosis. Ectopic expression of 4E-BP1 in nontransformed fibroblasts (CREF) had no discernible impact on the assembly of eIF4F or on cell viability. However, inhibition of phosphorylation with rapamycin led to disassembly of eIF4F and activation of apoptosis. In accord with this result, introduction of mutant forms of 4E-BP1 lacking specific phosphorylation sites into CREF also activated apoptosis. These findings suggest that the FRAP/mTOR kinase cascade, as well as other pathways leading to 4E-BP1 phosphorylation, is an important component of the antiapoptotic signaling system activated by extracellular survival factors. In addition, these data demonstrate a close connection between cellular functions controlled by 4E-BP1 and the regulation of apoptosis, thus supporting the concept that activation of cap-dependent translation leads to the synthesis of regulatory proteins that antagonize apoptosis.

A detailed examination of the relationship between repression of cap-dependent initiation and apoptosis proved informative. The mutant forms of 4E-BP1 studied suppressed cap-dependent initiation in exactly the rank order of potency expected based on prior publications (A70 > A65 > A37/A46 > wild type) (9, 10, 24, 25). In general, activation of apoptosis followed this same pattern, with A70 > A65 > wild type. Only A37/A46 deviated from expectation, a result with several important implications. First, despite being expressed at lower levels than the other 4E-BP1 forms, A37/A46 suppressed translation most robustly. This dissociates the level of ectopic protein expression from the intensity of translational repression and strongly suggests that our findings do not result from toxicity of the mutant proteins themselves. Second, introduction of the double mutant (A37/A46) allowed us to reduce cap-dependent translation initiation by 70%, which by itself was not sufficient to achieve maximum levels of 4E-BP1-induced apoptosis. This makes it unlikely that the apoptosis observed was triggered by metabolic poisoning due to inadequate protein synthesis—pointing strongly instead to a change in the balance of factors regulating apoptosis, not those required to sustain other critical physiological functions. Third, in cells expressing the A37/A46 mutant, translation via IRES was activated concomitantly with a decline in apoptosis. Somewhat surprisingly, rather than leading to potentiation of apoptosis as might be expected from the simplest model of translational control (that cap-dependent translation rescues, and

translation via IRES promotes, apoptosis), apoptosis was actually attenuated.

The data in this report together with the published literature lead us to speculate that at some threshold level, the proapoptotic effect of hypophosphorylated 4E-BP1 may be mitigated by concomitant activation of IRES-mediated translation of rescue proteins. In this connection, experimental precedent does exist for translation of known rescue moieties via IRES, including insulin-like growth factor I receptor, insulin-like growth factor II, and X-linked inhibitor of apoptosis protein (17). Along these lines, recent findings indicate that the potent mediator of IRES-dependent translation, p97/DAP5/NAT1, is a bifunctional regulator of neuroblastoma cell fate—essential for both viability and apoptosis (37). Alternatively, as repression of cap-dependent translation is intensified, apoptosis may be attenuated by a reduction in cap-dependent death proteins independent of any effects exerted by proteins translated using IRES.

Our prior studies (28, 29, 35) together with the data reported here establish the functional import of regulating cap-dependent translation initiation in the control of programmed cell death. In addition, as the process of apoptosis unfolds, the translational machinery itself is attacked, setting up potential positive or negative feedback loops. For example, eIF4GI and eIF4GII are cleaved by factors triggering, controlling, or executing apoptosis (5, 21), resulting in shutdown of cap-dependent protein synthesis. In addition to the eIF4G family proteins, 4E-BP1 is also cleaved during apoptosis (2, 36). In accord with the general concept that cap-dependent translation machinery is suppressed during apoptosis (2, 4), one of the 4E-BP1 daughter fragments retains the ability to sequester eIF4E (36). In this regard, recent work demonstrates that sequestration of eIF4E by overexpressed 4E-BP1 (28) or by synthetic oligopeptides that associate with its eIF4G–4E-BP1 binding motif (14) is proapoptotic. These findings indicate that the cap-dependent initiation apparatus is both a regulator and target of the apoptotic machinery and suggest that degradation of specific components of the initiation complex is an integral part of apoptotic death.

Based on current experimental evidence, it appears possible that cell fate is in part determined by a dynamic interplay between cap-dependent translation and translation via IRES. The level and activity of the 4E-BP family of proteins are major determinants of the rate of translation initiation utilizing eIF4F, the cap binding apparatus. Thus, this formulation assigns a central role to the 4E-BP family of proteins and the biochemical pathways governing their phosphorylation status in the control of apoptosis. It also highlights the need for studies in which cap-dependent and IRES-mediated translation is independently manipulated—to elucidate the rules governing which mechanism is utilized in the translation of critical mRNA species encoding proteins that regulate cell viability.

ACKNOWLEDGMENTS

This work was supported by the following grants: National Institutes of Health 2P50-HL50152 (P.B.B.), Department of Defense BC980414 (V.A.P.), Medical Research Council of Canada (N.S.), Medical Research Council of Canada Doctoral Award (A.-C.G.), and National Institutes of Health HL 07741-07 (S.L.).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 5, Issue of January 31, pp. 3015–3022, 2003
Printed in U.S.A.

Translation Factor eIF4E Rescues Cells from Myc-dependent Apoptosis by Inhibiting Cytochrome *c* Release*

Received for publication, August 28, 2002, and in revised form, November 15, 2002
Published, JBC Papers in Press, November 18, 2002, DOI 10.1074/jbc.M208821200

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Eukaryotic translation initiation factor 4E (eIF4E) markedly reduces cellular susceptibility to apoptosis. However, the mechanism by which the translation apparatus operates on the cellular apoptotic machinery remains uncertain. Here we show that eIF4E-mediated rescue from Myc-dependent apoptosis is accompanied by inhibition of mitochondrial cytochrome *c* release. Experiments achieving gain and loss of function demonstrate that eIF4E-mediated rescue is governed by pre-translational and translational activation of *bcl*-x as well as by additional intermediates acting directly on, or upstream of, the mitochondria. Thus, our data trace a pathway controlling apoptotic susceptibility that begins with the activity state of the protein synthesis machinery and leads to interdiction of the apoptotic program at the mitochondrial checkpoint.

The integration of extracellular information by a cell into a decision to live or die is of fundamental importance during development, wound healing, immune responses, and tumorigenesis (1, 2). Most studies addressing the regulation of apoptosis have focused on transcriptional control, signal transduction, and other post-translational regulatory events. More recently, it has become evident that apoptosis is also subject to translational control. Cap-dependent translation involves the assembly of initiation factors at the 5′ mRNA terminus to form the trimolecular cap binding complex, eIF4F. These factors include the cap-binding protein, eIF4E,[1] an ATP-dependent RNA helicase, eIF4A, and an eIF4G polypeptide (eIF4GI or eIF4GII), which serves as a docking site for eIF4E and eIF4A (3). The function of eIF4E is negatively regulated by members of the translational repressor family, the eIF4E-binding proteins, that sequester eIF4E in a translationally inactive complex (4). Growth factors and other pro-survival stimuli promote phosphorylation of the eIF4E-binding proteins. Hyperphosphorylated eIF4E-binding protein has a decreased affinity for eIF4E, resulting in its liberation to initiate translation.

The activity of the cap-dependent translation initiation apparatus is a major determinant of cell fate during development (5, 6) and post-natal life (7). A constitutively active initiation apparatus sustains morphogenesis *ex vivo* (5), promotes cell cycle transit (8, 9), and leads to malignant transformation (9–12), whereas repression of aberrant translation initiation reverses oncogenesis (13–15). We have previously shown that apoptosis can be governed by the activity of eIF4E. Overexpression of eIF4E rescues cells from apoptosis (16, 17), whereas sequestration of eIF4E by overexpressed eIF4E-binding protein 1, triggers apoptosis and markedly diminishes tumorigenesis (18, 19).

The apoptotic program can be triggered through at least two distinct signaling pathways with the potential for cross-talk. One pathway, leading to activation of caspase-8, is triggered by ligation of specific cell surface death receptors, such as Fas/CD95 or tumor necrosis factor α receptor (20). The second pathway, initiated by various stressors such as cytotoxic drugs and radiation, is transduced through a caspase-2-mediated series of steps into mitochondrial release of cytochrome *c* (21). Subsequent formation of the apoptosome, a complex containing cytochrome *c*, adapter protein Apaf-1, and procaspase-9 leads to activation of caspase-9 (22). When activated, caspases-8 and -9 activate effectors caspases-3, -6, and/or 7, which in turn cleave critical cellular targets, resulting in death (23). Proteins of the Bcl-2 family tightly regulate mitochondrial release of cytochrome *c*. Proapoptotic proteins, such as Bid, Bax, Bad, and Bak, form pores in the outer mitochondrial membrane, whereas the anti-apoptotic proteins, Bcl-2 and Bcl-X_L, inhibit pore formation (24, 25). In most cell types, these two pathways converge, and receptor-induced activation of caspase-8 also results in mitochondrial release of death promoters with subsequent activation of the apoptosome-dependent caspase cascade (26). Thus, the mitochondria integrate a variety of cell death signals, and the ability of Bcl-2/Bcl-X_L to interdict apoptosis is one hallmark of mitochondrial involvement in the apoptotic pathway (27, 28).

Despite the strong connection between translational control and apoptosis, little is known about the underlying mechanisms. We have previously demonstrated that over-expressed eIF4E averts Myc-dependent apoptosis, at least in part, through a cyclin D1-dependent process (17). To provide further insight into the mechanism of eIF4E rescue, we examined which steps in the apoptotic cascade were blocked by eIF4E in rat embryo fibroblasts sensitized to apoptosis by constitutive expression of c-Myc. Here we show that cells rescued by eIF4E neither release cytochrome *c* from their mitochondria nor do they activate any downstream steps in the apoptotic cascade. A survey of Bcl-2 family members in rescued fibroblasts revealed most to be in the basal state. However, Bcl-X_L was dramatically

* This work was supported by Department of Defense Grant BC98414 (to V. A. P.), National Institutes of Health Grant 2P50-HL50152 (to P. B. B.), and National Institutes of Health Grant HL 07741-07 (to S. L. and D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: eIF4E, eukaryotic translation initiation factor 4E; REF, rat embryonic fibroblasts; PBS, phosphate-buffered saline; PARP, anti-poly(ADP-ribose) polymerase; HPLC, high performance liquid chromatography; ASO, antisense oligodeoxynucleotide; UTR, untranslated region; IRES, internal ribosomal entry site; DAPI, 4,6-diamidino-2-phenylindole.

increased due to the selective recruitment of Bcl-X_L mRNA to ribosomes by eIF4E as well as from activation of pre-translational stages of Bcl-X_L production. Experiments achieving gain and loss of Bcl-X_L function indicated that factors other than Bcl-X_L are involved in eIF4E-dependent blockade of cytochrome *c* release. These data provide the first direct link between translationally mediated antiapoptotic signaling and the apoptotic machinery and underscore the pleotropic nature of survival signaling downstream of up-regulated eIF4E.

MATERIALS AND METHODS

Cell Lines—Cell lines were derived from rat embryonic fibroblasts (REF) as described (17). REF/Myc cells, which overexpressed c-Myc, were provided by Dr. Weinberg (Whitehead Institute, Cambridge, MA). REF/Myc/4E cells were generated by transducing REF/Myc with retrovirus CRE-BCS encoding wild-type murine eIF4E (retrovirus was kindly provided by P. Leboulch, Massachusetts Institute of Technology, Cambridge, MA). Both REF/Myc and REF/Myc/4E cells were grown in complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4.5 g/liter glucose, 100 units/ml penicillin, 100 units/ml streptomycin, and 250 ng/ml amphotericin).

Analysis of Apoptosis—Cells were subjected to proapoptotic conditions as specified in the text and figure legends. Both adherent and detached cells were collected, fixed in 70% ethanol, washed with PBS, and stained with propidium iodide stain mixture (50 μg/ml of propidium iodide, 0.1% Triton X-100, 32 μg/ml EDTA, 2.5 μg/ml RNase in PBS) for 45 min at 37 °C. DNA content was determined by quantitative flow cytometry using the CellQuest program.

Immunoblot Analysis—Cultured cells were rinsed in PBS, trypsinized, and collected by centrifugation. Cells were resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton-X, 1% sodium deoxycholate) supplemented with protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, aprotinin, and pepstatin A) and microcentrifuged at 12,000 × *g* for 10 min, and the supernatant was retained. The protein concentration was measured using the Pierce BCA protein assay kit. 20 μg of total protein was resolved by 15% SDS/PAGE, followed by immunoblotting with the following antibodies: anti-Bcl-X_L (Trevigen), anti-Bcl-2 (Trevigen), anti-Bax (Santa Cruz Biotechnology), anti-Bad (Santa Cruz Biotechnology), anti-actin (Sigma), anti-tubulin (Sigma), anti-caspase-3 (Santa Cruz Biotechnology), anti-poly(ADP-ribose) polymerase (PARP; Upstate Biotechnology). Cytochrome *c* was analyzed by subjecting 50 μg of cell free extract to 12% SDS/PAGE, transferred to nitrocellulose membranes, and incubated with mouse anti-cytochrome *c* antibody (BD Biosciences). Blots were incubated with an appropriate horseradish peroxidase-coupled secondary antibody and detected by ECL (Amersham Biosciences).

Caspase Activity Assay—Cells were subjected to proapoptotic conditions, and both detached and adherent cells were collected, incubated with lysis buffer, and centrifuged for 5 min at 10,000 × *g*. Active caspase-3 was measured using a CleavaLite caspase-3 activity assay kit (Chemicon). 50 μg of lysate was added to a CleavaLite Renilla luciferase bioluminescent substrate that contains the caspase-3 cleavage site, DEVD. After incubation for 1 h at 37 °C, fresh luciferase substrate was added, and luminescence was read in a Lumat luminometer (EG&G Berthold). For caspase-9 activity, the cell lysate was centrifuged at 10,000 × *g* for 1 min, supernatant was collected, and caspase-9 activity was determined by cleavage of LEHD-*p*-nitroanilide using a caspase-9 colorimetric assay (R&D systems).

Determination of Cytochrome c Subcellular Distribution—Adherent and floating cells were pooled and suspended in mitochondrial buffer (250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 μg/ml pepstatin A). Cells were mechanically lysed using a loose-fitting Wheaton cell homogenizer, and centrifuged at 1000 × *g* for 10 min to remove nuclei and unbroken cells. The supernatant was centrifuged at 13,000 × *g* for 20 min to pellet the mitochondria. The resulting supernatant (cytosolic fraction) was centrifuged at 100,000 × *g* for 1 h. Samples were diluted to obtain a total protein concentration of 0.4 μg/μl, and cytochrome *c* concentration in each sample was determined by enzyme-linked immunosorbent assay using an R&D systems Quantikine murine cytochrome *c* immunoassay kit.

Measurement of Mitochondrial Membrane Potential—Cells were seeded on glass cover slips precoated with fetal calf serum and incubated (24 h at 37 °C) in Dulbecco's modified Eagle's medium, 10% fetal calf serum. Cultures were continued with or without 5 μM lovastatin for an additional 24 h. Mitochondria were stained by exposing cells to 0.5 μg/ml rhodamine 123 dye for 45 min at 37 °C. Medium was changed to PBS immediately before analyzing the staining pattern of the cells.

Polyribosome Preparation—15 plates of actively proliferating cells in 100-mm dishes were treated with cycloheximide (100 μg/ml) for 5 min, harvested by trypsinization, and lysed as described (50) using 60 strokes in a Dounce homogenizer. Lysate was centrifuged at 10,000 × *g* for 10 min, and the nuclei pellet was removed. Cytoplasmic extract (1.5 mg measured at A_{260}) was layered onto a 5-ml, 0.5–1.5 M sucrose gradient. The sucrose gradients were centrifuged at 200,000 × *g* in a Beckman SW50 rotor for 90 min at 4 °C. The gradients were fractionated using an ISCO density gradient fractionator monitoring absorbance at 254 nm. Five 1-ml fractions were collected from each sample into tubes containing 100 μl of 10% SDS.

Quantification of bcl-x mRNA by Real-time PCR—The RNA from each fraction of the sucrose gradient was extracted using Tri-reagent (Sigma) and quantitated. RNA (5 μg) from each fraction was treated with DNase I (DNA-free™, Ambion, Austin) according to the manufacturer's directions. cDNA was synthesized from 2 μg of each RNA sample with a Taqman reverse transcriptase reagent kit (Applied Biosystems) primed with oligo-dT. Rat *bcl*-x DNA sequences for upper (5′-GGAGACCCCCAGTGCCATCAAT-3′) and lower (5′-AGTGCCCCGCCAAAGGAGAAA-3′) primers and rat *bcl*-2 DNA sequences for upper (5′-CACCCCTGGCATCTTCTCCTTCC-3′) and lower (5′-GCATCCCAGCCTCCGTTATCCT-3′) primers were selected using the DNA STAR program (DNASTAR, Inc., Madison, WI), and the resulting sequences were synthesized in the University of Minnesota microchemical facility and purified by HPLC. Real time PCR was performed using a LightCycler FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals). The LightCycler PCR protocol consisted of a 10-min denaturation followed by 42 cycles of 95 °C for 10 s, 68 °C for 5 s, and 72 °C for 16 s. Reactions were set up as recommended by the manufacturer, optimized with 4 μM $MgCl_2$ with each primer at 1 μM. A portion of the cDNA reaction (9.6 μl) was used for amplification of each gradient fraction. Quantification of mRNA was carried out by comparison (linear interpolation) of the number of cycles to saturation in each sample, with the number of cycles in a concurrently run standard containing a known amount of target mRNA (5 concentrations of the standard were used which spanned the range of values for the samples).

Northern Blot Analysis—Total RNA was isolated using an RNeasy Total RNA kit (Qiagen, Santa Clarita, CA), and 15 μg RNA/lane was electrophoresed through 1% agarose, 2.2 M formaldehyde gel and transferred to nylon filters. A synthetic 40-mer oligonucleotide (5′-GGTGGTCATTCAGGTAGGTGGCCATCCAACTTGCAATCCG-3′), which was designed specifically to recognize *bcl*-X_L (2), was 3′ end-labeled with 50 μCi of [α-^{32}P]dATP (3000 Ci/mmol) using terminal deoxynucleotidyltransferase (Roche Molecular Biochemicals). A glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech, Palo Alto, CA) was random-primed with 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol). Radiolabeled probes were purified using Sephadex G-25 spin columns (Roche Molecular Biochemicals). Hybridization was performed in 50% (v/v) formamide, 5× sodium chloride/sodium citrate (SSC), 1% SDS, 1× Denhardt's, 10% dextran sulfate, and 250 μg/ml yeast RNA (Sigma) with either the 3′ end-labeled *bcl*-X_L oligonucleotide probe or the random primer-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe at 42 °C overnight. Blots were washed twice in 0.1× SSC, 0.1% SDS at 50 °C for 30 min and exposed to x-ray film.

Messenger RNA Stability—RNA stability was quantified as described previously (29). To assess stability of *bcl*-x mRNA, cells were treated with 5 μg/ml actinomycin D (Sigma) for the time intervals specified in the text and figure legends. Total RNA was isolated and analyzed by Northern blot.

Generation of REF/Myc/Bcl-X_L Clonal Cell Lines—REF/Myc cells were stably transfected with a pAPuro vector containing an 800-bp fragment encoding wild type Bcl-X_L (a gift from Dr. E. Prochownik, Children's Hospital, Pittsburgh, PA) using the FuGENE 6 (Roche Diagnostics) transfection technique. Selection of transfected cells was begun after 24 h in complete medium containing 4 μg/ml puromycin. Resistant clones were isolated after 12–16 days.

Bcl-X_L Antisense Oligonucleotides—Phosphorothioate antisense and nonsense DNA oligodeoxynucleotides were synthesized and purified by HPLC (Operon Technologies, Inc., Alameda, CA). An 18-mer antisense oligodeoxynucleotide sequence spanning the translation start codon of *bcl*-x mRNA and a control scrambled sequence were (*a*) antisense 5′-CCG GTT GCT CTG AGA CAT-3′ and (*b*) scramble 5′-CTG AAC GGA GAG ACC CTT-3′. Cells were seeded into chambers of 8-well glass chamber slides overnight and shifted to Dulbecco's modified Eagle's

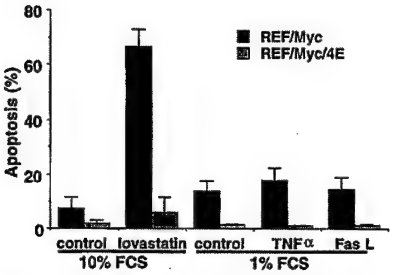

FIG. 1. **eIF4E rescues cells from Myc-dependent apoptosis.** REF/Myc and REF/Myc/4E cells were cultured in complete medium with or without 5 μM lovastatin for 24 h or in Dulbecco's modified Eagle's medium plus 1% fetal calf serum (*FCS*) alone, with 100 ng/ml tumor necrosis factor-α (*TNF-α*), or with 100 ng/ml Fas ligand (*Fas-L*) for 24 h. Apoptosis was quantified by flow cytometry. The data presented are the mean (±S.E.) of four independent experiments.

medium containing 0.1% fetal calf serum with or without 5 μM lovastatin, 40 μM oligodeoxynucleotides, or both. Cells were cultivated for 72 h with one media change. Cells were fixed with ice-cold 70% ethanol and stained with acridine orange, and apoptosis was quantified using morphological criteria, as described (16, 17).

Cytochrome c Immunostaining—Cells were seeded at a density of $5 \times 10^3/cm^2$ in 24-well clusters onto glass cover slips and cultured for 24 h in complete medium. Cultures were either continued in complete medium alone or containing 5 μM lovastatin for 18 or 24 h. Cells were rinsed with PBS and fixed in PBS containing 4% paraformaldehyde. The fixed cells were incubated in blocking buffer (PBS containing 5% normal goat serum, 1% bovine serum albumin, and 0.3% Triton X-100) for 30 min and for an additional 2 h in PBS containing 1% bovine serum albumin, 1% normal goat serum, and 1 μg of anti-cytochrome *c* antibody (Promega) per ml. Cells were washed 3 times in PBS and incubated for 30 min in PBS containing 1% bovine serum albumin, 1% normal goat serum, and 1 μg of fluorescein isothiocyanate-conjugated anti-mouse antibody (Sigma) per ml. Cells were rinsed 3 times in PBS, nuclei-stained with DAPI (0.1 mg/ml), and cover slips were mounted onto slides.

RESULTS

Increased Expression of eIF4E Inhibits Death Receptor-mediated and Stressor-induced Apoptotic Pathways—We previously reported that ectopic expression of eIF4E rescues REF harboring constitutively expressed c-*myc* (REF/Myc) from apoptosis triggered by either serum restriction (16) or cytotoxic stress (17). To evaluate whether eIF4E also suppresses death receptor-mediated apoptosis, we examined its impact on REF/Myc cell viability after treatment with tumor necrosis factor family ligands. When eIF4E was ectopically overexpressed in REF/Myc cells (REF/Myc/4E), apoptosis was inhibited in response to each apoptotic trigger tested (Fig. 1), indicating that eIF4E promoted suppression of the death receptor-mediated as well as the stress-induced apoptotic cascades. However, the magnitude of kill in response to tumor necrosis factor-α or Fas ligand was modest and not amenable to further study. In contrast, more than 64% of cells underwent apoptotic death in response to lovastatin, a value reduced to nearly the basal frequency by ectopic expression of eIF4E. Based on this result, we restricted our subsequent studies of eIF4E rescue to lovastatin-induced apoptosis.

Ectopic Expression of eIF4E Blocks Release of Cytochrome c from Mitochondria—The cleavage of PARP into an 85-kDa daughter fragment is a downstream step common to most forms of apoptosis. We have traced the apoptotic pathway upstream of PARP cleavage in both REF/Myc and REF/Myc/4E cells (Fig. 2). Treatment with lovastatin led to the appearance of the 85-kDa fragment of PARP in REF/Myc cells but not in REF/Myc/4E cells (Fig. 2*A*). Similarly, the proform of caspase-3 (32 kDa) was cleaved into its active 17-kDa form in REF/Myc but not in REF/Myc/4E (Fig. 2*B*). Consistent with this observation, REF/Myc cells manifested a progressive increase in

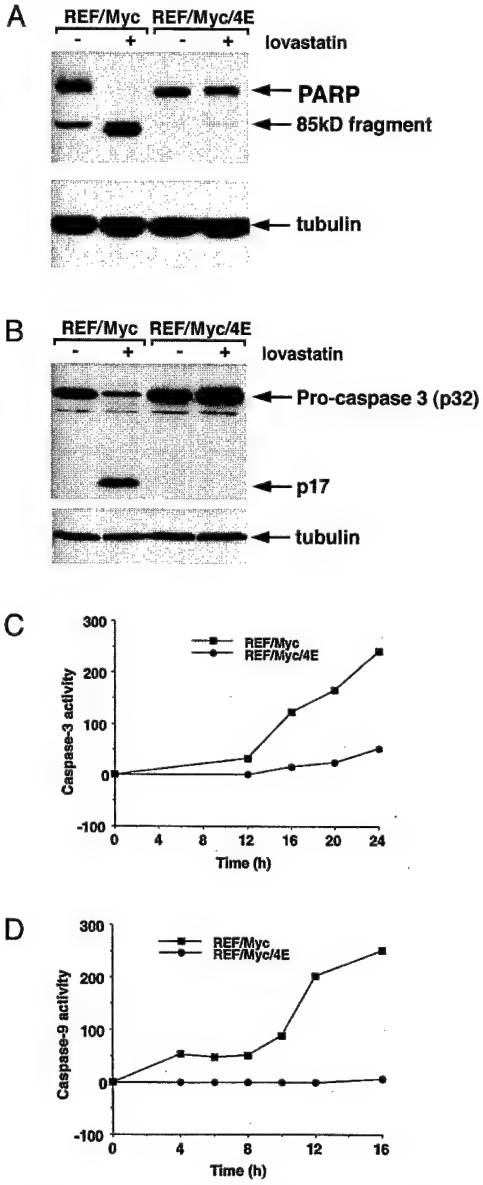

FIG. 2. **eIF4E blocks PARP cleavage and suppresses caspase-3 and caspase-9 activation.** *A* and *B*, immunoblot analysis of PARP and caspase-3. REF/Myc and REF/Myc/4E cells were cultured in complete medium with or without 5 μM lovastatin for 24 h. Cleavage of PARP (*A*) and activated caspase-3 (*B*) were detected by immunoblot analysis with tubulin shown as a loading control. *C* and *D*, analysis of caspase-3 and caspase-9 enzymatic activity. REF/Myc or REF/Myc/4E cells were cultured for the time interval indicated in the presence of 5 μM lovastatin. Caspase-3 like activity was detected using CleavaLiteTM caspase-3 activity assay kit (*C*), and caspase-9 like activity was detected with a colorimetric assay (*D*); results shown are representative of three independent experiments.

caspase-3 activation, whereas caspase-3 activity remained low in REF/Myc/4E cells throughout the entire interval of observation (Fig. 2*C*). Caspase-9 followed the same pattern. Activation was noted as early as 4 h in REF/Myc cells, reaching levels more than 3-fold that of control by 16 h (Fig. 2*D*). In REF/Myc/4E cells caspase-9 remained in the inactive state.

Cytochrome *c* normally resides between the inner and outer mitochondrial membranes and is not usually found in the cytoplasm (30). Because cytochrome *c* is required for activation of caspase-9 (31), we examined the subcellular distribution of cytochrome *c* after exposure of cells to lovastatin. At base line, cytochrome *c* was detectable at low levels in the cytoplasm of REF/Myc cells, a value that increased more than 3-fold after

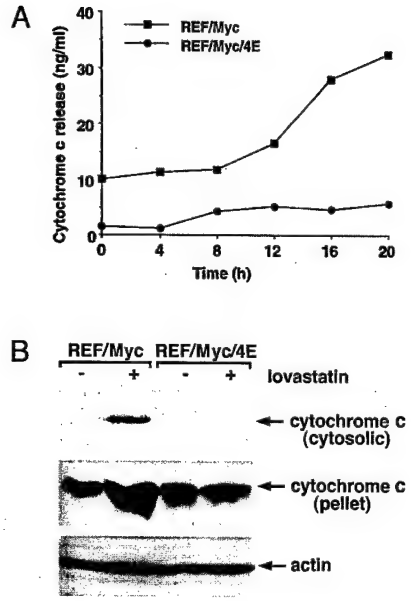

FIG. 3. **eIF4E prevents release of cytochrome *c* from the mitochondria to the cytosol.** *A*, immunoassay of cytoplasmic cytochrome *c*. REF/Myc or REF/Myc/4E cells were cultured in complete medium with 5 μM lovastatin for the time intervals indicated. Release of cytochrome *c* from mitochondria was determined by quantifying cytochrome *c* concentration in the cytoplasm by enzyme-linked immunosorbent assay. Data shown are representative of three independent experiments. *B*, immunoblot analysis of cytochrome *c* subcellular distribution. REF/Myc or REF/Myc/4E cells were cultured in complete medium with or without 5 μM lovastatin for 24 h. Cytochrome *c* was detected in cell extracts (cytosolic fraction and pellet) by immunoblot analysis (with actin shown as a loading control).

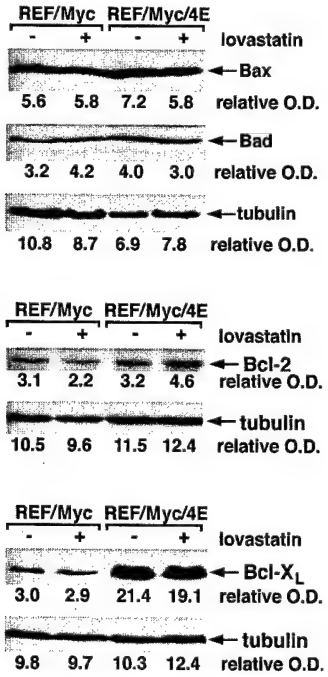

FIG. 4. **eIF4E increases steady state levels of Bcl-X_L.** REF/Myc or REF/Myc/4E cells were cultured in complete medium with or without 5 μM lovastatin for 24 h. Immunoblot analysis was carried out for Bcl-2 family members, pro-apoptotic, Bax and Bad (*upper panel*), and anti-apoptotic, Bcl-2 (*middle panel*) and Bcl-X_L (*lower panel*), with tubulin used as a loading control. Shown below each blot is densitometric quantification expressed in arbitrary units. *O.D.*, optical density.

20 h of lovastatin treatment (Fig. 3*A*). In contrast, cytochrome *c* remained near basal levels in the cytoplasm of REF/Myc/4E cells after lovastatin treatment, a result corroborated by immunoblot analysis (Fig. 3*B*).

Mitochondrial inner transmembrane potential collapse frequently precedes cytochrome *c* release and caspase activation (32). To examine whether this was the case in our system, we treated cells with lovastatin for 24 h and measured the uptake of rhodamine 123, a cationic fluorophore that enters mitochondria in direct proportion to membrane potential (33). REF/Myc and REF/Myc/4E cells took up the dye similarly after lovastatin treatment, suggesting that the suppression of cytochrome *c* release in REF/Myc/4E cells was not due to eIF4E-induced alterations in mitochondrial membrane depolarization (data not shown).

Overexpressed eIF4E Selectively Stimulates Expression of Bcl-X_L—Deregulated c-Myc mediates apoptosis in some transformed cell lines by selectively decreasing the mRNA and protein levels of the death antagonists Bcl-2 and Bcl-X_L (34, 35). We therefore examined whether eIF4E had any effect on this interplay between c-Myc and the Bcl-2 family proteins. Immunoblot analysis demonstrated that overexpression of eIF4E resulted in a 7-fold increase of Bcl-X_L protein in REF/Myc cells without significantly affecting the expression levels of Bcl-2, Bax, or Bad (Fig. 4).

Overexpression of eIF4E Increases Recruitment of Bcl-X_L mRNA to Ribosomes—To examine whether overexpression of eIF4E increased cellular levels of Bcl-X_L protein by direct translational activation, total RNA from REF/Myc and REF/Myc/4E was stratified by sucrose gradient centrifugation to separate translationally active transcripts (more bound ribosomes resulting in more rapid transit through the gradient) from less translationally active transcripts. The resulting fractions were subjected to quantitative PCR analysis for the *bcl*-X_L transcript, comparing it to the *bcl*-2 transcript as a control. Examination of the absorbance pattern revealed a bias toward heavier polyribosomes, with an increased proportion of RNA in fractions 4 and 5 in cells ectopically expressing eIF4E (Fig. 5*A*). Real time PCR quantification indicated that the transcript for *bcl*-2 was distributed similarly in REF/Myc and REF/Myc/4E cells, with fraction 2 (the least translationally active) and fraction 5 (the most translationally active) containing similar amounts of transcript (Fig. 5*B*). In marked contrast, there was a significant increase in the quantity of *bcl*-X_L mRNA appearing in heavy polyribosomes in REF/Myc/4E cells (Fig. 5*C*). The average number of bound ribosomes per bcl-X_L transcript in REF/Myc/4E cells was 3.7 compared with 1.5 in REF/Myc cells, indicating a 2.5-fold increase in the rate of bcl-X_L mRNA translation initiation (36, 37). Thus, one mechanism for the increase in Bcl-X_L protein was direct translational activation of its mRNA.

Overexpressed eIF4E Increases Cellular Levels of Bcl-X_L mRNA—A growing number of studies show that eIF4E directly or indirectly participates in a variety of pre-translational stages of protein synthesis including production and processing of mRNA and its nuclear-cytoplasmic transport (38). To gain insight into the influence of eIF4E on pretranslational events in the synthesis of Bcl-X_L, we measured the abundance and stability of its mRNA. Quantitative PCR analysis demonstrated a dramatic effect of eIF4E on steady state levels of *bcl*-X_L mRNA but not on the levels of *bcl*-2 mRNA (Fig. 6*A*). The level of *bcl*-X_L mRNA was increased nearly 30-fold in REF/Myc/4E cells, a result supported by Northern blot analysis (Fig. 6*B*). The kinetics of *bcl*-X_L mRNA degradation were only marginally altered by ectopic eIF4E (half-life of 3 h for REF/Myc and 2.5 h for REF/Myc/4E; Fig. 6*C*). These data indicate that in addition to direct translational activation, ectopic eIF4E stimulates synthesis of Bcl-X_L by increasing the abundance of its transcript.

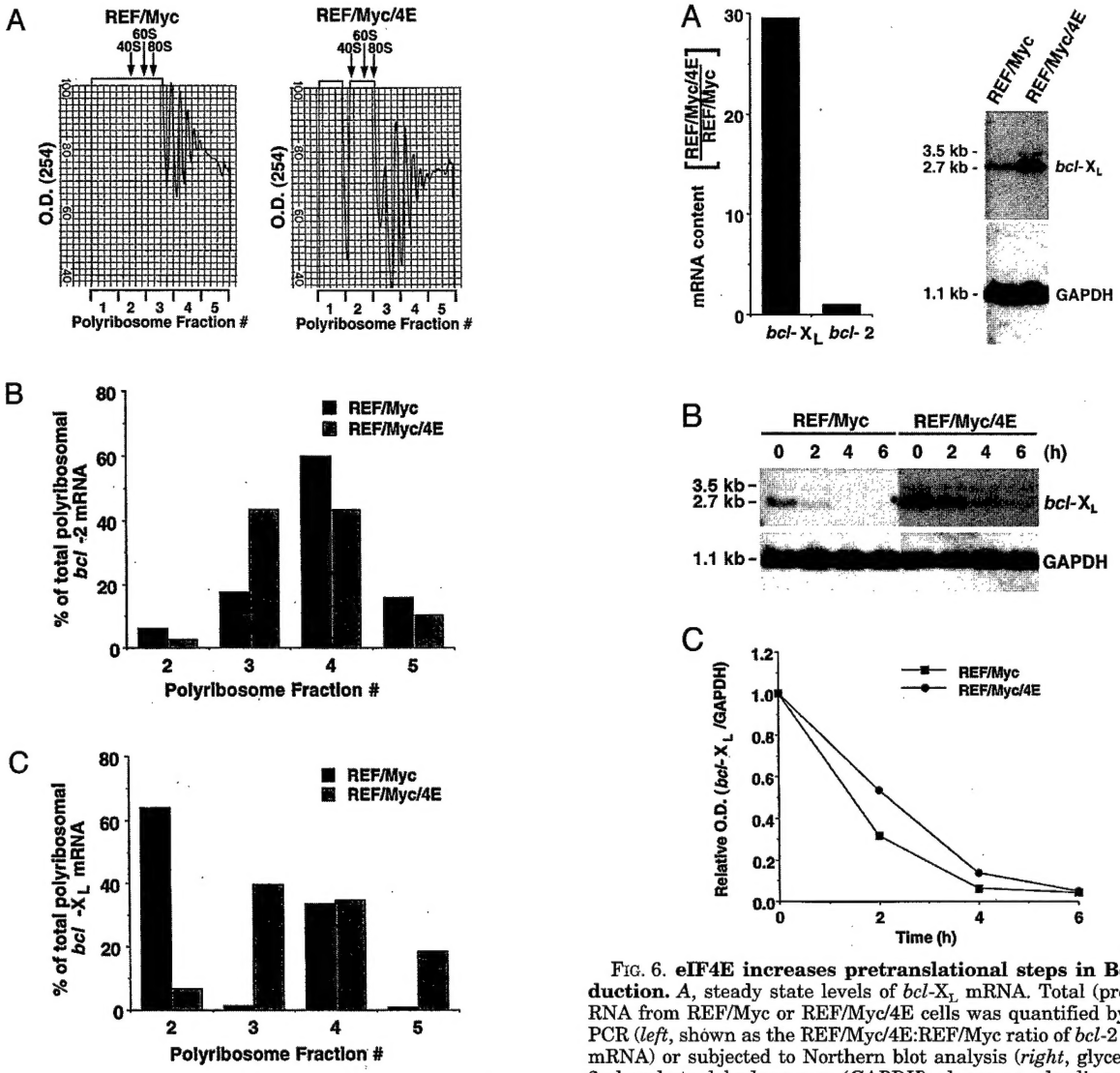

FIG. 5. **eIF4E increases recruitment of *bcl*-X_L transcripts to ribosomes.** *A*, polyribosome analysis. Polyribosomes from REF/Myc or REF/Myc/4E cells were resolved into discrete fractions by sucrose gradient ultracentrifugation. Shown is the optical density (*O.D. (254 nm)*) as a function of sedimentation velocity with the heavier polyribosomes corresponding to the higher numbered fractions. The position of 40, 60, and 80 S ribosomal fractions (designated with *arrows*) were off scale. *B* and *C*, translational activity of *bcl*-2 and *bcl*-X_L mRNA. RNA from each gradient fraction was reverse-transcribed and quantified by real time PCR. A standard curve was generated using five concentrations of a cell extract containing a known quantity of target mRNA that spanned the range of mRNA content in the samples to be analyzed. Quantification of *bcl*-2 and *bcl*-X_L mRNA in REF/Myc and REF/Myc/4E cells was carried out by linear interpolation. Shown is the polysome distribution of *bcl*-2 (*B*) and *bcl*-X_L (*C*) mRNA in REF/Myc and REF/Myc/4E as a function of position in the gradient.

FIG. 6. **eIF4E increases pretranslational steps in Bcl-X_L production.** *A*, steady state levels of *bcl*-X_L mRNA. Total (pre-gradient) RNA from REF/Myc or REF/Myc/4E cells was quantified by real time PCR (*left*, shown as the REF/Myc/4E:REF/Myc ratio of *bcl*-2 and *bcl*-X_L mRNA) or subjected to Northern blot analysis (*right*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), shown as a loading control). *B*, stability of *bcl*-X_L mRNA. REF/Myc or REF/Myc/4E cells were treated with 5 μg/ml actinomycin D for the time interval indicated. Total RNA was isolated and analyzed by Northern blot. *C*, shown is RNA stability expressed as the optical density (*O.D.*) ratio of Bcl-X_L and glyceraldehyde-3-phosphate dehydrogenase.

Decreasing Cellular Levels of Bcl-X_L Reduces eIF4E-mediated Rescue from Apoptosis—To assess whether increased expression of Bcl-X_L is required for the antiapoptotic function of eIF4E, REF/Myc/4E cells were treated with an antisense oligodeoxynucleotide (ASO) directed against human *bcl*-X_L mRNA sequences in the predicted translation initiation region. A scrambled oligonucleotide served as a control. Incubation of cells with the ASO for 72 h significantly reduced the level of Bcl-X_L protein, whereas the scrambled oligonucleotide had no effect (Fig. 7*A*). Continuation of these cultures for an additional 24 h in the presence of lovastatin led to a significant increase in the apoptotic frequency of ASO compared with the scrambled oligonucleotide control (Fig. 7*B*). However, despite reduction of Bcl-X_L in REF/Myc/4E by ASO below levels found in REF/Myc, the degree of apoptosis was only half (62.5% in REF/Myc *versus* 30% in ASO-treated REF/Myc/4E). These data indicate that increased expression of Bcl-X_L is necessary for the full-scale antiapoptotic function of ectopic eIF4E and that Bcl-X_L is not the sole determinant of eIF4E-mediated rescue from apoptosis.

Ectopic Expression of Bcl-X_L Does Not Fully Reproduce eIF4E Rescue—To determine whether increased expression of Bcl-X_L could substitute for eIF4E in the rescue of REF/Myc cells from apoptosis, we generated clonal lines of REF/Myc cells stably expressing a range of Bcl-X_L protein. Twelve puromycin-resistant clones derived from REF/Myc cells transfected with a pAPuro/*bcl*-x vector were screened for levels of Bcl-X_L expression. Two clonal cell lines were selected for further investigation (Fig. 8*A*), one line with a Bcl-X_L level that closely matched the level of Bcl-X_L expression in REF/Myc/4E cells (clone 7) and a second line in which the level of Bcl-X_L was increased more than 5-fold (clone 13).

To validate the function of our construct, we first examined the kinetics of cytochrome *c* release in the clonal lines by immunoblot and immunolocalization. Ectopic expression of

eIF4E completely suppressed cytochrome *c* release in the basal state and after lovastatin treatment (Fig. 8*B*). In the basal state, 15% of the cytochrome *c* in REF/Myc cells was in the cytoplasm, whereas after ectopic expression of Bcl-X_L, virtually no cytoplasmic cytochrome *c* was detected. After treatment with lovastatin, levels of cytochrome *c* in the cytoplasm of REF/Myc cells were stable for 12 h, gradually increasing to more than 40% of total cellular cytochrome *c* by 24 h (Fig. 8*B*). For the Bcl-X_L clonal lines, there was a steady translocation of cytochrome *c* from the mitochondria to the cytoplasm beginning at 8 h and increasing up to 24 h. At all time points examined, the magnitude of cytochrome *c* release in cells overexpressing Bcl-X_L was significantly less than that observed in REF/Myc, with a rank order of potency reflecting the expression level of Bcl-X_L (Fig. 8*B*).

Morphological analysis showed a perinuclear granular pattern of cytochrome *c* in REF/Myc, REF/Myc/Bcl-X_L 7, REF/Myc/Bcl-X_L 13, and REF/Myc/4E cells in the basal state, consistent with a mitochondrial subcellular localization (Fig. 8*C*). After lovastatin treatment for 18 h, cytochrome *c* acquired a diffuse distribution (consistent with cytoplasmic localization) in REF/Myc and REF/Myc/Bcl-X_L 7, and after 24 h a diffuse pattern was evident in REF/Myc/Bcl-X_L 13. In contrast, the cytochrome *c* signal remained punctate in REF/Myc/4E cells for the 24-h observation interval. Consistent with these results, ectopic expression of Bcl-X_L significantly attenuated apoptosis in REF/Myc cells (Fig. 8*D*). However, even the highest expressing clonal line manifested an apoptotic frequency of 30%, more than 2-fold that observed in REF/Myc/4E. These data indicate a qualitative difference between rescue by eIF4E and rescue by Bcl-X_L.

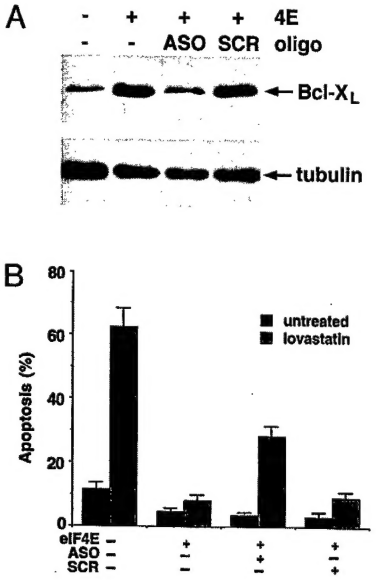

FIG. 7. **Decreasing Bcl-X_L to base-line levels does not fully restore the apoptotic response in REF/Myc/4E cells.** *A*, analysis of Bcl-X_L protein levels after treatment with *bcl*-X_L antisense oligodeoxynucleotides. REF/Myc/4E cells were treated with either 40 μM *bcl*-X_L ASO or a scramble control (*SCR*) for 72 h. ASO was directed against human *bcl*-X_L mRNA sequences in the predicted translation initiation region to inhibit synthesis of Bcl-X_L protein, which was detected by immunoblotting. Expression of tubulin is shown as a loading control. *B*, apoptosis. REF/Myc/4E cells were treated as in *A* and cultured for an additional 24 h in the presence or absence of 5 μM lovastatin. The proportion of cells with hypodiploid DNA content was quantitated by flow cytometry.

DISCUSSION

To begin deciphering the rules governing translational control of cell death, in this report we examined which components of the apoptotic machinery were inhibited when apoptosis was suppressed by ectopic overexpression of eIF4E using Myc-dependent apoptosis in fibroblasts as a model. Here we show that eIF4E rescues cells by blocking release of cytochrome *c* from the mitochondria. Rescue by eIF4E was mediated in part by its ability to increase cellular levels of Bcl-X_L, a key apoptotic antagonist. The eIF4E-induced increase in Bcl-X_L was robust, occurring through at least two separate mechanisms, 1) direct

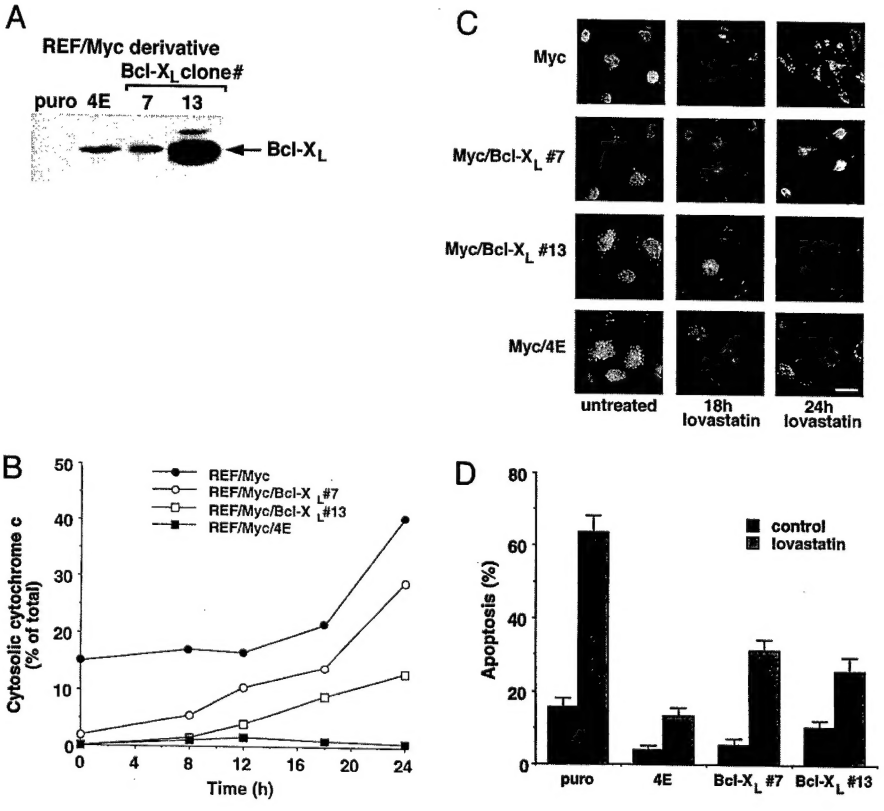

FIG. 8. **Increasing steady state levels of Bcl-X_L attenuates but does not eliminate apoptosis in REF/Myc cells.** *A*, steady state levels of Bcl-X_L in clonal REF/Myc cell lines stably transfected with Bcl-X_L. REF/Myc cells were stably transfected with Bcl-X_L, and a series of clonal lines were developed. Shown is the immunoblot analysis of Bcl-X_L protein in parental cells, clone 7 (chosen to closely match the Bcl-X_L level in REF/Myc/4E) and clone 13 (chosen to have a level of Bcl-X_L in great excess to that in REF/Myc/4E). *B* and *C*, subcellular distribution of cytochrome *c*. REF/Myc, REF/Myc/Bcl-X_L 7, and REF/Myc/Bcl-X_L 13 cells were cultured in complete medium with 5 μM lovastatin for the time intervals indicated. Shown are the relative amounts of cytosolic cytochrome *c* determined by immunoblot (*B*) and the cellular distribution of cytochrome *c* by immunofluoresence (nuclei stained with 4,6-diamidino-2-phenylindole; representative micrographs from three independent experiments are shown). The *scale bar* equals 20 μM (*C*). *D*, apoptosis. Clonal lines of REF/Myc cells ectopically expressing Bcl-X_L or empty puromycin vector were cultured for 24 h in the presence or absence of 5 μM lovastatin. Apoptosis is shown for each cell line, quantified by flow cytometry.

translational activation and 2) increase in the abundance of the *bcl*-x_L transcript. However, gain and loss of function experiments indicated that Bcl-X_L did not fully account for the potent antiapoptotic activity of eIF4E. These observations indicate that the set point for cellular susceptibility to apoptosis can be governed by translational control.

The rate of protein synthesis is intimately connected with the process of programmed cell death. An ordered shut down of protein synthesis is one of the earliest events during apoptosis, and suppression of global translation can enhance apoptosis (37–40). Although maintenance of global translation tends to antagonize apoptosis, cap-dependent and IRES-driven modes of translation can impact cell death differently. One mediator of IRES-regulated translation, death-associated protein 5 (DAP5/p97/NAT1), for example, promotes cell death (39, 40). In contrast, eIF4E, the principal activator of cap-dependent translation, rescues cells from apoptosis (16–18).

Our finding that eIF4E modulates the mitochondrial checkpoint for apoptosis provides a glimpse into the mechanism by which the translational apparatus links survival signaling to the apoptotic machinery. We show that Bcl-X_L is regulated by direct control its translation, a finding in accord with a recent publication reporting that fibroblast growth factor-2 translationally activates both Bcl-X_L and Bcl-2 through a Ras/MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase)-dependent signaling pathway (41). In this regard, eIF4E is known to be activated in a MEK-dependent manner (42), thus defining a putative survival pathway from growth factor receptors through Ras activation of the translational apparatus to the Bcl-X_L/Bcl-2-regulated apoptotic checkpoint.

What is the mechanism by which eIF4E selectively increases translation of the Bcl-X_L transcript? It is generally agreed that activated eIF4E preferentially stimulates translation of those mRNA with a high degree of complexity in their 5′-untranslated region (5′-UTR), such as those with upstream open reading frames or a high GC-content (43, 44). One documented example of translational control of Bcl-2 family proteins through a 5′-UTR is the *bcl*-2 mRNA, which contains a 35-bp upstream open reading frame (45). The 5′-UTR of the *bcl*-X_L message is relatively long and highly GC-rich (46), suggesting that it too may be a good candidate for eIF4E-mediated regulation, which we found to be the case.

Pretranslational stages of Bcl-X_L production were also activated by overexpressed eIF4E, suggesting that transcriptional regulation, posttranscriptional maturation, and nuclear export of its mRNA may all be targets of translational control. Transcriptional control of *bcl*-x is an established mechanism for regulating cellular levels of Bcl-X_L (47, 48). A number of transcription factors such as Stat5 (49, 50), NFκB (51), PAX3 (52), and Ets2 (53) may all be involved in regulating *bcl*-x gene expression. Although we speculate that eIF4E might increase the level of *bcl*-X_L mRNA by activating translation of these factors or their regulators, detailed studies of the 5′- and 3′-UTR of the transcript will be needed to clarify the mechanism. Our attempts to separately quantify the impact of pre-translational and translational activation of Bcl-X_L production by eIF4E using actinomycin D were unsuccessful due to the short half-life of its mRNA.[2] It should be pointed out, however, that at least two transcription factors, c-Fos and c-Myc, are known to be targets for translation control (54). Future studies will undoubtedly expand the list.

Bcl-X_L is not the sole mediator of eIF4E-dependent antiapoptotic signaling upstream of mitochondria, since gain of Bcl-X_L function could not reproduce the robust effects of eIF4E on apoptosis nor could loss of Bcl-X_L function completely abrogate eIF4E mediated rescue, thus supporting the available literature that translational control of apoptosis is mediated by a set of antiapoptotic effectors (24, 54, 55). Because the vast majority of mRNAs encoding growth factors, their cognate receptors and signal transduction pathways have long 5′-UTRs or contain upstream open reading frames, it is likely that a plethora of antiapoptotic pathways emanate from activated eIF4E. In this connection, we have previously documented that one downstream effector of eIF4E, cyclin D1, is required for eIF4E-mediated rescue from Myc-dependent apoptosis (17). Future investigations will be required to establish whether cyclin D1 and Bcl-X_L lie on the same or different survival pathways downstream of eIF4E.

Our data help to clarify how c-Myc and eIF4E cooperate in tumorigenesis. Deregulated c-Myc triggers uncontrolled cell cycle progression and apoptosis, processes that have opposite effects on oncogenesis (56). In this regard, the oncogenic potential of c-Myc, which is masked by its own pro-apoptotic potency, requires an additional event that antagonizes apoptosis (57). Overexpressed eIF4E effectively cooperates with c-Myc in malignant conversion of rodent fibroblasts (10, 58). Our previous reports (16, 17) and present findings suggest that the impact of eIF4E on Myc-dependent oncogenesis is determined to a significant degree by its ability to suppress the mitochondrial events essential for Myc-activated apoptosis.

Thus, our data trace a novel pathway that controls cell susceptibility to apoptosis. The pathway originates at the protein synthesis machinery and leads to pretranslational and translational modification of the apoptotic program at the mitochondrial checkpoint. Our findings highlight the need for developing new discovery tools that accurately identify those transcripts that are translationally activated by eIF4E to mitigate a proapoptotic stress. In preliminary studies, combining polyribosome preparations to stratify mRNA by the number of bound ribosomes with gene expression microarray has begun to show promise. Independent of the identity of the entire set of translationally activated messages mediating eIF4E rescue, our data begin to explain how the translation initiation apparatus functions to suppress apoptosis and promote oncogenesis, suggesting a plausible mechanism to explain the dramatic oncogenic synergy between eIF4E and a variety of pre-neoplastic alterations that promote both uncontrolled cell cycle progression and cell death.

Acknowledgments—We thank Dr. Janet Dubinsky (Department of Neuroscience, University of Minnesota) for helping to detect the mitochondrial transmembrane potential using rhodamine 123 dye. We also thank Drs. Nahum Sonenberg, Ronald Jemmerson, and Timothy Behrens for invaluable help and discussions and Dr. Edward Prochownik (Children's Hospital, Pittsburgh, PA) for the pAPuro/*bcl*-X vector.

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